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The Intermediary Metabolism of the Mammary Gland

1. RESPIRATION OF LACTATING MAMMARY GLAND SLICES IN PRESENCE OF CARBOHYDRATES

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(*Received* 3 *February* 1949)

Studies of the respiratory exchange of the mammary gland have hitherto been confined to ruminants in which the arterio-venous method can conveniently be applied to the udder. Though blood velocity data, which are difficult to obtain, are necessary to convert arterio-venous differences in oxygen and carbon dioxide into absolute values for uptake and output respectively, the respiratory quotient (R.Q.) can be obtained without blood-flow measurements.

Results in the goat (Graham, Houchin, Peterson & Turner, 1938; Reineke, Stonecipher & Turner, 1941), and cow (Petersen & Shaw, 1942; Shaw, 1946) point to an R.Q. greater than unity for the lactating udder, which has been interpreted (Graham *et al.* 1938; Reineke, Stonecipher & Turner, 1941) as indicating the formation of milk fat, more specifically of the short chain fatty acids (Reineke, Stonecipher & Turner, 1941), from carbohydrate.

In many of the above series of determinations, at any rate those on non-anaesthetized animals, the individual values were rather variable, and indeed the high variance of their results occasioned comment by Petersen & Shaw (1942). However, a small series of determinations on cows in this laboratory (Bottomley & Folley, unpublished results), in which the blood samples were protected against gas exchange during and after collection by use of the apparatus of Austin, Cullen, Hastings, McLean, Peters & Van Slyke (1922), were satisfactorily uniform, giving a mean R.Q. $= 1{\cdot}25 \pm 0{\cdot}12$ (95% fiducial limits) in cases where no haemoconcentration, indicative of manipulative disturbance of the subject, occurred during blood sampling. When significant haemoconcentration occurred the R.Q. was usually less than unity.

Working on anaesthetized goats, to minimize the risk of disturbance during blood sampling, Reineke, Stonecipher & Turner (1941) obtained a fairly uniform series of values, but the mean R.Q. (1·09) could hardly be considered significantly greater than unity, though it was raised to 1·18 by applying, following the precedent of Graham *et al.* (1938), a correction for CO_2 believed to be used for the synthesis of urea by the udder. As regards anaesthetized cows, a series of values obtained by Shaw (1946), which gave a mean R.Q. of 1·27, was perhaps not as uniform as would be desirable.

In sum it might fairly be said that while there is a certain amount of evidence suggesting a high R.Q. for the lactating ruminant udder *in vivo*, it is by no means as clear cut and conclusive as might be desired, particularly in view of criticisms which have been levelled against the arterio-venous technique as applied to the udder (Kay, 1947; Folley, 1949). Because of this, and because R.Q. values reported for the perfused isolated bovine udder have been less than unity (Shaw, 1939) or hardly significantly greater (Petersen & Shaw, 1942), an investigation of the respiratory metabolism of the lactating mammary gland by the tissue-slice method appeared to be of interest. This was the more so because, although mammary gland slices have been used for studying lactose formation *in vitro* (Grant, 1935, 1936; Knodt & Petersen, 1945), the only *in vitro* study of the respiration of mammary tissue known to us is that of Kleiber, Smith & Levy (1943), who used 'portions' of mammary tissue which evidently were not slices in the accepted sense, and who did not determine R.Q. The tissue-slice method has the advantages that it is applicable to all mammals, thus enabling the ruminant udder to be compared with the mamma of the non-ruminant, and it can be used for studies of phases of intermediary metabolism which are hardly open to attack by the arterio-venous method. The results of an investigation of the respiration of mammary gland slices from various species, metabolizing carbohydrates, are given in the present paper. Part of this work has already been published in preliminary form (Folley & French, 1948*a*, *b*).

METHODS

Animals. Most of the experiments were carried out on mammary tissue from rats, two strains of which were used, Wistar and hooded Norway. The majority were autopsied at the height of lactation (15th–21st days) as were also most of the mice (Swiss strain) used. Groups of rabbits (Dutch) were killed on the 7th and 25th–28th days of lactation, and guinea pigs on the 10th–12th days. The animals were killed by dislocation of the spinal cord and mammary tissue immediately removed, the litters being allowed to suckle right up to the time of death.

Udder tissue was obtained from lactating goats, mostly crossbreds bought in, but in a few cases from pedigree British Saanens of the Institute herd. Usually the goats were shot

and the tissue removed immediately; in three cases pieces of tissue for slicing were removed from living goats under *cyclo*propane anaesthesia. The acquisition of authentic lactating udder tissue from cows giving adequate yields of milk presents much more formidable difficulties since cows are not normally slaughtered while in milk. We were, however, able to obtain tissue from a few cows culled from the herds of the Institute or the University of Reading, four of them giving about 10 lb. of milk daily and all milked regularly up to slaughter.

Mammary gland slices. In the case of rats, mice and rabbits, abdominal* mammary glands were taken, except in one experiment on rats (see p. 119) in which only thoracic glands were available. Because of the structure of mammary tissue, which consists of lobules of alveoli bounded by septa of tough, elastic connective tissue, suitable thin slices of lactating mammary gland are difficult to cut by the usual methods. It was, however, possible to cut satisfactory slices less than 0·4 mm. thick by means of the microtome of Stadie & Riggs (1944). On removal from the microtome the slices, if straightened out by forceps on a flat surface, are seen to be of such irregular, lacy form, consisting of small pieces (presumably lobules) of alveolar tissue attached to a network of connective strands, that the area cannot be measured, thus precluding determination of thickness by the usual method. The more or less constant slice thickness given by the microtome was therefore determined as usual on liver slices cut under similar conditions. A slice thickness of 0·4 mm. or less seems to be necessary for work with mammary gland since slices about 0·6 mm. thick give a lower Q_{O_2}.

Quotients (Q_{O_2}, Q_{CO_2}, Q_{acid}) were calculated in μl./mg. final dry wt./hr.; they were not calculated on the basis of initial dry weights, as recommended by many workers, because lactating mammary tissue retains considerable quantities of milk in the alveoli (Folley & Greenbaum, 1947). Milk is leached out from the slice during a manometric experiment, as evidenced by the increasing opacity of the medium, and although no attempt has been made to determine the final milk content it seems unlikely that much would remain in a thin slice under these conditions. The final dry weights of the slices were thus used for the calculation of the quotients, and errors due to the presence of small amounts of milk at the end of the experiments would tend to compensate for those due to loss of tissue fragments during shaking. In actual fact, mammary gland slices, though looking rather fragile and indeed tattered, did not show any tendency to break up during the experiments.

Cow udder slices were cut at the abattoir, placed in Ringer-bicarbonate (gassed with 95% O_2, 5% CO_2) at room temperature and at once brought to the laboratory. The extra delay from animal to manometric flask was about 10 min.

Duplicate determinations on different slices from the same piece of tissue mostly showed satisfactory agreement, though occasionally there were larger differences (e.g. Fig. 3), probably due to the presence of different proportions of parenchyma and stroma in the slices. The presence of non-secretory tissue, as well as the above-mentioned 'milk error', would not affect R.Q., but would cause some underestimation of other quotients.

Manometric methods. All experiments were done at 37°. R.Q., Q_{O_2} and $Q^{O_2}_{acid}$ were determined by the method of Dickens & Šimer (1931*a*) over 3 hr. using Dickens & Greville (1933*b*) flasks. The medium was the Ringer-bicarbonate of Krebs & Henseleit (1932) and the gas mixture 95% O_2, 5% CO_2. In some experiments O_2 uptake was measured in Ringer-phosphate using ordinary Warburg flasks and pure O_2. For determinations of anaerobic acid production the gas mixture was 95% N_2, 5% CO_2 passed over hot Cu foil. The Warburg vessels contained 2·5 ml. of Ringer-bicarbonate and were gassed for 15 min. before the taps were closed.

With few exceptions determinations were in duplicate. In most experiments one or two slices (total 80–100 mg.) were used, but in the case of bovine udder tissue it was necessary to use more tissue to get a sufficient rate of gas exchange.

RESULTS

Rate of oxygen uptake

Typical experiments on gland from a fully lactating rat are shown in Figs. 1 and 2. The tissue shows an appreciable O_2 uptake in Ringer-phosphate (pH 7·4) in absence of substrate, and the rate falls off gradually over 3 hr. With glucose (0·2%) present from the outset the respiration rate was much higher and linear over 3 hr. (Fig. 1), while if the glucose was added from a side arm after 60 min. the tissue could still respond with an unmistakable enhancement in uptake (Fig. 2). The marked effect of glucose on the respiration rate of this tissue is in accord with the relative lack of reserve carbohydrate, as evidenced by our unpublished observations of the low glycogen content of rat mammary gland (see Folley, 1949) and by the non-utilization of lactose (see p. 120).

Respiration of rat mammary gland in presence of glucose

The results obtained with rat mammary gland in the presence of glucose are summarized in Table 1. The fully lactating gland, i.e. at 15–21 days of lactation under laboratory conditions (see below), shows in the presence of glucose a fairly high Q_{O_2} (9–10) which is almost double the endogenous value. Analysis of data obtained at different stages of lactation, mostly to be presented in a subsequent paper, indicated no great change in Q_{O_2} over the period 15–21 days, so that data for rats killed within this period have been pooled throughout.

The question of the amount of substrate needed for 3 hr. experiments on mammary tissue needs consideration, because this tissue may utilize glucose for synthesis of lactose, and possibly fat, as well as for oxidation. The effect of glucose concentrations varying from 0·1 to 1·0% was therefore investigated in a series of experiments in which the effect of each glucose concentration was compared with that of 0·2%, the standard concentration adopted at the

* In this paper the word 'abdominal' as applied to mammary glands refers to the two abdominal and four inguinal glands.

outset of this work, on duplicate slices from the same animal. There was no evidence that glucose concentrations greater than 0·2 % were more effective in increasing the total respiration over 3 hr. (see Table 1 for results with 0·2 and 0·3 % glucose respectively), but in order to allow an ample margin, 0·3 % glucose was adopted for the later experiments. Results with 0·2 and 0·3 % glucose (or other carbohydrate) were pooled whenever necessary in compiling the Tables given in this paper.

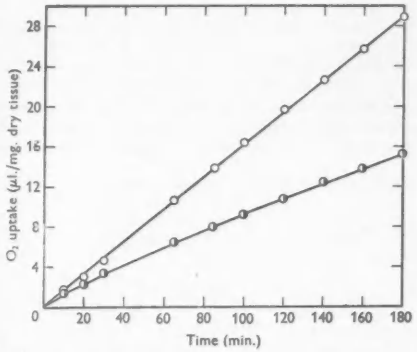

Fig. 1. Oxygen uptake of mammary gland slices from rat on nineteenth day of lactation. Upper curve: 0·2 % glucose present from the outset; lower curve: no substrate. Temp. 37°; pH 7·4; phosphate buffer; atmosphere, 100 % O_2.

Table 1 includes results for a group of rats in which slices were cut from thoracic glands because the abdominal glands were required for another purpose. We consider the thoracic glands to be less suitable for metabolic work than the abdominal glands because they tend to be less suckled, and therefore might lactate less actively, and because they are interlaced with strands of muscle tissue. Nevertheless, the mean Q_{O_2} was, if anything, slightly higher than for abdominal glands (Table 1).

Combining the data for three groups of rats killed at 15–21 days (Table 1), the mean $-Q_{O_2}$ for thirty-nine rats is 9·3 ± 0·2 (Table 2). The results (Table 1) show some indication that the Q_{O_2} in early lactation (3–6 days) is lower than during the last third of the 21-day lactation period, as was found by Kleiber *et al.* (1943) provided that their results were calculated on a dry tissue basis. These observations led us to make the above-mentioned detailed study of changes in the respiration of mammary tissue at various stages of lactation.

The results (Table 1) show clearly an unusually high R.Q. for rat mammary slices at the height of lactation, the mean value for the thirty-nine rats killed at 15–21 days being 1·53 ± 0·02 (see Table 2). This happens to be double the value (0·77 ± 0·03, for ten rats) obtained in the absence of substrate (Table 1). Laser (1942) has criticized the validity of R.Q. determinations on tissue slices over periods as long as 3 hr. However, there seems to be no reason to doubt the accuracy of our values on these grounds, since in experiments with rat mammary gland slices in which the curve expressing the resultant of O_2 uptake and CO_2 output (the 'composite curve' of Elliott, Greig & Benoy, 1937) was studied over 3 hr.,

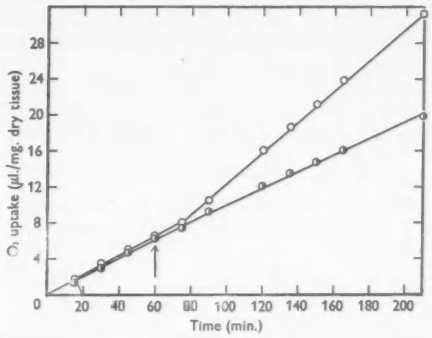

Fig. 2. Oxygen uptake of mammary gland slices from rat on nineteenth day of lactation. Upper curve: 0·2 % glucose added from side arm at arrow; lower curve: no substrate. Temp. 37°; pH 7·4; phosphate buffer; atmosphere, 100 % O_2.

Table 1. *Respiration of lactating rat mammary gland slices in presence of glucose*

No. of rats	Stage of lactation at autopsy (days)	Substrate	$-Q_{O_2}$ (μl./mg. final dry wt./hr.) Mean*	Range	R.Q. Mean	Range
2	6 and 12	Nil	—	4·5, 6·5	—	0·73, 0·68
10	15–21	Nil	5·3 ± 0·3	4·1–7·8	0·77 ± 0·03	0·66–0·92
3	3–6	Glucose (0·2 %)	5·6	3·6–7·7	1·47	1·37–1·66
20	15–21	Glucose (0·2 %)	9·1 ± 0·3	6·8–11·5	1·53 ± 0·03	1·31–1·86
8†	17	Glucose (0·2 %)	9·9 ± 0·4	7·8–11·2	1·51 ± 0·03	1·33–1·67
11	15–21	Glucose (0·3 %)	9·3 ± 0·4	6·3–11·9	1·55 ± 0·03	1·41–1·73
3	22–25	Glucose (0·2 or 0·3 %)	9·3	6·9–11·1	1·29	1·11–1·44

* In this and subsequent Tables results are shown as mean ± S.E.M. where appropriate.
† Slices from thoracic glands; all others from abdominal glands.

CO_2 output exceeded O_2 uptake from the outset, and the curves were linear over most of the period only showing a slight falling off towards the end. This latter point would indicate that our R.Q.'s were if anything underestimated.

Respiration of rat mammary gland slices in presence of various sugars

The respiration of mammary gland slices from rats killed at 15–21 days of lactation was investigated in the presence of a number of carbohydrates. In all experiments substrate concentrations were 0·2 or 0·3 %, except in the case of lactose which was used in 0·4 or 0·6 % concentration. The results are given in Table 2.

Of the sugars studied, which include those known to be of outstanding importance in mammalian biochemistry, mannose was the only one besides glucose to be utilized by lactating mammary gland slices, as evidenced by a Q_{O_2} equal to that shown in the presence of glucose and by an R.Q. greater than unity. In a few cases (lactose, fructose, sorbose), in which the endogenous respiration was studied on duplicate slices, Q_{O_2} was not increased in presence of the sugar.

Table 2. *Respiration of lactating rat mammary gland slices in presence of various carbohydrates*

(All rats killed between 15 and 21 days of lactation and all slices taken from abdominal glands except in eight of the experiments with glucose in which thoracic tissue was used. Substrate concentrations 0·2 or 0·3 %, except for lactose which was 0·4 or 0·6 %.)

		$-Q_{O_2}$		R.Q.	
Substrate	No. of exps.	Absolute (μl./mg. final dry wt./hr.)	Percentage of value given by another slice from the same animal in presence of glucose	Absolute	Percentage of value given by another slice from the same animal in presence of glucose
D-Glucose	39	9·3±0·2	—	1·53±0·02	—
D-Galactose	3	6·3	61	0·78	58
D-Mannose	4	9·7	96	1·55	102
D-Fructose	3	5·9	71	0·78	53
L-Sorbose	1	6·8	69	0·63	39
D-Glucose-1-phosphate	1	7·2	59	0·96	69
N-Acetylglucosamine	1	4·4	44	0·89	57
Glucosamine	1	6·4	62	0·84	53
L-Arabinose	1	6·9	68	0·68	46
D-Arabinose	1	6·0	48	0·78	46
D-Ribose	1	4·9	53	0·72	42
D-Xylose	1	4·5	36	0·77	46
L-Rhamnose	1	6·0	70	0·80	50
Lactose	3	5·4	65	0·79	57
Maltose	1	6·5	80	0·85	57
Cellobiose	1	5·8	72	0·73	49
Sucrose	1	5·5	53	0·82	52
Raffinose	1	4·1	48	0·88	55
Glycogen	1	6·7	62	0·70	43

Respiration of mammary tissue of various species in presence of glucose

In studying the respiration of lactating mammary tissue from various species the question arose when to kill the animals so as to obtain fully lactating tissue. Since the course of lactation in the other common laboratory mammals, with the possible exception of the mouse, is less well known than in the rat, some representatives of each form (mouse, guinea pig, rabbit) were taken early in lactation, but after milk secretion could be considered as well established, while others were taken some days later (see Table 3) but, it was believed, before lactation began to decline because of gradual self weaning of the young. In the case of the mouse, the second group were killed at 15 days by analogy with the rat and it seems probable that these glands were in full lactation. The guinea pig presented most difficulty because its young are capable of fending for themselves at birth so that the demands on, and functional response of, the mammary gland in the guinea pig under laboratory conditions are quite problematical.

For the goats, most of which were purchased (in milk), the stages of lactation were unknown save in four cases, all of which were in fairly advanced lactation. Since lactating goats bought in the autumn, as these were, are most likely to have kidded during the previous February or March, the results may be taken as typical for the udder of the goat after eight or more months' lactation. With three of the four goats (pedigree animals from the Institute herd) whose kidding dates were known, mammary tissue was removed under *cyclo*propane anaesthesia. The results for these are given separately in Table 3, although there is no evidence that the anaesthetic affected the issue. Of the six cows available, all save two were of necessity in rather advanced lactation, although four were yielding approximately 10 lb. milk/day at slaughter. In all cases the substrate was 0·3 % glucose except for some rats where 0·2 % was used.

The results are summarized in Table 3, which indicates that Q_{O_2} is inversely correlated with body size, mouse being the most active tissue studied ($-Q_{O_2} = 16{\cdot}0$) and cow the least ($-Q_{O_2} = 3{\cdot}5$). As with the rat, R.Q. was significantly greater than unity in

Table 3. *Respiration of lactating mammary gland slices from various species in presence of glucose**

Animal	Stage of lactation	No. of animals	$-Q_{O_2}$ (μl./mg. final dry wt./hr.) Mean	$-Q_{O_2}$ (μl./mg. final dry wt./hr.) Range	R.Q. Mean	R.Q. Range
Mouse	5 days	3	10·5	8·3–12·0	1·65	1·52–1·80
	15 days	6	16·0±1·0	13·3–19·0	1·94±0·07	1·66–2·11
Rat	15–21 days	39	9·3±0·2	6·3–11·9	1·53±0·02	1·31–1·86
Guinea pig	5 days	1	9·6	—	1·06	—
	10–12 days	5	9·0±0·4	8·3–10·0	1·17±0·06	1·01–1·32
Rabbit	7 days	6	6·6±0·3	5·2–7·2	1·45±0·05	1·28–1·60
	25–28 days	3	5·8	3·6–8·2	1·32	1·21–1·52
Goat	Unknown	8	4·6±0·3	3·2–5·7	0·86±0·03	0·69–0·95
	Advanced†	3	5·7	5·0–6·2	0·85	0·74–0·93
Cow	Milk yield approx. 10 lb./day	4	3·5±0·5	2·2–4·7	0·80±0·04	0·64–0·93
	Milk yield < 5 lb./day	2	—	1·6, 1·9	—	0·87, 0·90

* Glucose concentration 0·3% except in the case of some rats where 0·2% was used.
† Tissue removed under anaesthesia.

Table 4. *Acid production by lactating mammary gland slices*

Animal	Stage of lactation (days)	Substrate	No. of exps.	Q_{acid} (μl./mg. final dry wt./hr.) Mean	Q_{acid} (μl./mg. final dry wt./hr.) Range
		Aerobic*			
Mouse	15	Glucose (0·3%)	6	1·7±0·3	0·9–2·4
Rat	15–21	Nil	10	0·6±0·1	0·2–1·0
Rat	15–21	Glucose (0·2 or 0·3%)	39	1·7±0·1	0·8–2·9
Guinea pig	10–12	Glucose (0·3%)	5	0·7±0·1	0·3–0·9
Rabbit	7	Glucose (0·3%)	6	1·4±0·3	0·7–2·9
Goat	Unknown	Glucose (0·3%)	8	1·6±0·1	1·2–2·2
Cow	Various stages	Glucose (0·3%)	6	1·7±0·2	1·0–2·3
		Anaerobic†			
Rat	15–21	Glucose (0·3%)	6	9·6±1·3	7·3–13·8

* Calculated over 3 hr. † Calculated over initial 30 min.

the mouse, guinea pig and rabbit; but it was less than unity in the ruminants, cow and goat. Of the non-ruminants, mouse tissue gave the highest R.Q., the value being not significantly less than 2.

Acid production of lactating mammary gland slices

Values for the aerobic acid production obtained in 3 hr. experiments on tissue from various species are given in Table 4. Under aerobic conditions in bicarbonate medium the acid production is not unduly high by comparison with other tissues (Dickens & Šimer, 1931*b*; Dickens & Greville, 1933*a*), and there are no prominent species differences. The actual glycolysis may be even lower, since part at least of the acid production of mammary tissue may be due to formation of citric acid (Knodt & Petersen, 1946) or to synthesis of fatty acids.

Anaerobic experiments were carried out on rat tissue only. Typical experiments in which the rate of anaerobic acid production over 3 hr. periods was studied are illustrated in Fig. 3. The initially high rate of acid production falls off steadily over the whole period; hence the values for $Q^{N}_{CO_2}$ given in Table 4 were calculated from data for the initial 30 min. during which the rate of acid production is nearly linear. The reason for this steady falling off in acid production under anaerobic conditions is not known; exhaustion of the substrate was not responsible because the addition of extra glucose did not prevent it. It was not due to tissue damage caused by lowering of the pH of the medium, since in the experiment in which most acid was produced, the pH only fell by 0·2 unit over 3 hr. As in the case of aerobic acid production, it is possible that not all of the acid produced under anaerobic conditions is due to glycolysis. Nevertheless, it seems safe to

conclude from these results that mammary tissue exhibits a considerable Pasteur effect. The curves in Fig. 3 also illustrate the variation to be expected between duplicate slices from the same rat, and the lowered acid production in absence of substrate.

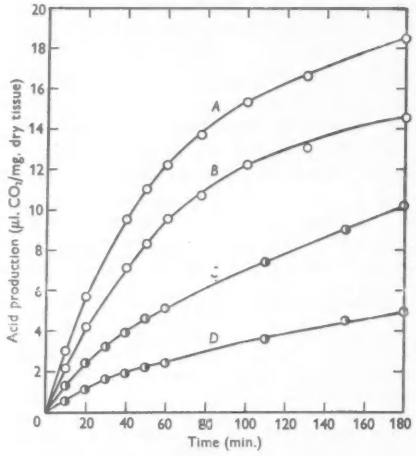

Fig. 3. Anaerobic acid production of slices of lactating rat mammary gland. Curves *A* and *B* relate to duplicate slices from a rat on the fifteenth day of lactation, both in presence of 0·3% glucose. Curves *C* and *D* relate to duplicate slices from a rat on the twenty-first day of lactation, *C* with 0·3% glucose, *D* with no substrate. Temp. 37°; pH 7·4; Ringer-bicarbonate; atmosphere, 95% N_2, 5% CO_2.

Effect of reduced food intake and adrenalectomy on the respiration of mammary tissue in the rat

The relation between the intensity of lactation and the respiratory metabolism of mammary tissue is of interest, and it was felt that a study of procedures which depress lactation, such as underfeeding or adrenalectomy, might provide preliminary information in this connexion. Thoracic mammary tissue was available for study from rats which had been adrenalectomized on the fourth day of lactation and also from sham-operated rats pair-fed to the reduced food intake of these, as well as from sham-operated rats fed *ad lib.* Details of the diet and general experimental procedure were as described for a similar experiment by Cowie & Folley (1948). All rats were killed on the seventeenth day of lactation and the slices were incubated with 0·2% glucose.

The results are given in Table 5. Lactation was depressed in both adrenalectomized and pair-fed sham-operated rats, markedly in the former and less so in the latter, as indicated by the litter-growth indices (Cowie & Folley, 1947). It is important to note that despite this the adrenalectomized rats and the pair-fed controls were still suckling young at the time of autopsy. Q_{O_2} was significantly depressed, and, notwithstanding the difference in lactational performance, to about the same extent in tissue from the adrenalectomized rats and from the pair-fed controls, while on the other hand, the aerobic acid production was significantly raised. Particularly striking is the fact that in tissue from both these groups the R.Q. was reduced to a value not far from unity. The possibility that, since the experiment was relatively long term, the mammae of the two groups under consideration had undergone some degree of involution by the time of autopsy must obviously be considered. However, Folley & Greenbaum (1948), in a discussion of the effects of these procedures on the mammary gland arginase levels, concluded that this was unlikely.

The results indicate that a period of partial inanition significantly lowers the Q_{O_2} of lactating mammary tissue, increases the apparent aerobic glycolysis and also depresses the high R.Q. to values near unity, i.e. to values intermediate between those given by fully lactating tissue in presence and absence of glucose respectively. Moreover, it would appear that adrenalectomy *per se* has no effect on the respiratory metabolism of the mammary gland.

Table 5. *Respiration and apparent aerobic glycolysis of slices of lactating rat mammary gland from adrenalectomized and pair-fed rats*

(All rats operated upon on the fourth day of lactation and killed on the seventeenth day. Respiration measurements made at 37° in presence of 0·2% glucose in Ringer-bicarbonate, pH 7·4.)

Treatment	No. of rats	Mean food intake from 4 to 17 days (g./day)	Litter-growth index*	$-Q_{O_2}$ (μl./mg. final dry wt./hr.) Mean	$-Q_{O_2}$ (μl./mg. final dry wt./hr.) Range	R.Q. Mean	R.Q. Range	$Q^{O_2}_{acid}$ (μl./mg. final dry wt./hr.) Mean	$Q^{O_2}_{acid}$ (μl./mg. final dry wt./hr.) Range
Sham operation: fed *ad lib.*	8	36·7±1·02	13·0	9·9±0·4	7·8–11·2	1·51±0·03	1·33–1·67	1·8±0·2	1·2–2·6
Sham operation: pair-fed	9	24·1±0·65	11·2	8·0±0·3	6·0–8·9	1·05±0·04	0·85–1·24	4·4±0·4	2·3–6·9
Adrenalectomy on fourth day of lactation: fed *ad lib.*	9		6·1	7·6±0·3	5·7–8·8	0·95±0·06	0·70–1·24	3·0±0·3	1·7–4·5

* See Cowie & Folley (1947).

DISCUSSION

The results presented in this paper show that, as might be expected from its outstanding synthetic activity, lactating mammary tissue respires fairly actively *in vitro* by comparison with other tissues. In the rat its activity is much less than that of nervous tissues or kidney cortex, but is of the same order as liver (Dickens & Šimer, 1931*b*; Dickens & Greville, 1933*a*). Our Q_{O_2} values for the rat are considerably higher than those given by Kleiber *et al.* (1943), but since these workers did not use thin slices it seems possible that their tissue was not equilibrated with O_2. Unlike some tissues, for example liver and kidney, the reserves of oxidizable substrates present in mammary tissue are low, since Q_{O_2} is markedly increased over endogenous values in the presence of glucose, but the effect is not comparable with that shown by nervous tissues (Dickens & Greville, 1933*a*). The low R.Q. obtained in the absence of substrate suggests that under these conditions the slice is oxidizing fat and perhaps protein. An interesting inverse correlation between Q_{O_2} for mammary tissue and body size, among various animal forms, is clearly indicated by our results.

The anaerobic acid production of rat mammary gland is quite considerable, though not as high as nervous and embryonic tissues (Dickens & Greville, 1933*a*), but since mammary tissue may produce citric, and under some conditions, fatty acids, it is doubtful whether the acid production measured by us either in the presence or absence of O_2 is wholly ascribable to glycolysis. For that reason we have not considered it worth while to estimate the Pasteur effect quantitatively, but it seems safe to conclude from our results that it is considerable.

Our results serve to complicate somewhat the position regarding the mammary gland R.Q. The *in vivo* experiments, mentioned earlier, which indicated an R.Q. greater than unity for the lactating mammary gland, were of necessity performed on ruminants (cow and goat). In our *in vitro* experiments, while R.Q. values considerably greater than unity have been given by mammary tissue from the mouse, rat, guinea pig and rabbit, all experiments with tissue from the cow and goat indicate an R.Q. less than unity with glucose as substrate. It may be objected that our ruminant slices were taken from udders which had been in lactation for considerable periods, and which might in consequence have contained considerable proportions of cells not in a state of optimal functional activity. Against this, it may be stated that slices from many of the same udders (cow and goat) have given R.Q.'s greater than unity in presence of acetate (Folley & French, 1948*c*, 1949), a product of rumen fermentation which is almost certainly of great importance as a metabolite in the ruminant (Elsden & Phillipson, 1948).

The high R.Q. indicated by *in vivo* experiments for the ruminant udder has been interpreted (Graham *et al.* 1938; Reineke, Stonecipher & Turner, 1941) as pointing to the synthesis of fat from carbohydrate in the active mammary gland. While our *in vitro* determinations lend no support to this theory as far as the ruminant is concerned, we feel that, despite the need for caution in interpreting the R.Q. of even a single organ (e.g. Soskin, 1941), our results with mammary gland slices from non-ruminants strongly suggest the possibility of fat synthesis from carbohydrate in these non-ruminant glands. Indeed, the possibilities of using rat or mouse mammary gland, especially the latter in view of its exceptionally high R.Q., for *in vitro* studies of fat synthesis seem particularly attractive, since mammary gland seems to be practically unique among mammalian tissues in giving a high R.Q. in the presence of glucose *in vitro*, coupled with a considerable respiration and hence, presumably, an appreciable rate of synthesis. A high R.Q. has been reported for adipose tissue (Henle & Szpingier, 1936; Mirski, 1942), but the rate of gas exchange is so low as almost to preclude accurate measurement. As for the high *in vivo* R.Q. of the ruminant udder, we have pointed out (Folley & French, 1949) that it is consistent with the synthesis of fat from acetate, a process which ruminant udder slices appear capable of effecting *in vitro*.

The striking respiratory response of mammary gland slices to mannose as well as glucose, while other sugars are inert, may be of significance in connexion with the mechanism of lactose synthesis. Reineke, Williamson & Turner (1941) have reported a significant uptake of blood glycoprotein by the udder of the lactating goat. Blood glycoproteins are known to contain mannose which, our results suggest, would be available for any purpose for which the gland uses glucose. However, Grant (1935) found no evidence of synthesis of lactose from mannose by guinea-pig mammary gland slices, which he reported were capable of effecting the synthesis from glucose. Nevertheless, in view of recent evidence (Friedmann, 1949) that, aside from glucosamine, the carbohydrate moiety of serum glycoproteins consists of an equimolecular mannose-galactose complex, the question whether lactose, or part of it, is formed in the mammary gland by transformation of a mannose-galactoside arising from blood protein seems worth consideration, particularly since our results suggest that glucose and mannose are interconvertible in the mammary gland. The non-utilization of lactose, hardly surprising since the mammary gland would not be expected to secrete a carbohydrate which it could readily oxidize, indicates that the chain of reactions from glucose to lactose is not reversible, at any rate at an appreciable speed, under these conditions. Folley (1949), in proposing a possible scheme for the biological synthesis of lactose, has discussed

the possibility that the final step may be an irreversible dephosphorylation. The failure of the slices to oxidize galactose and fructose indicates that these sugars are not convertible to glucose by this tissue as they are by the liver. In respect of attack on fructose, mammary gland differs from brain, retina, testis and kidney, all of which oxidize fructose (Dickens & Greville, 1933*a*). The non-utilization of glucose-1-phosphate may be due, as suggested by Grant (1936), to the inability of sugar phosphates to penetrate into the mammary gland cell.

The effects of long-continued partial inanition on the lactating mammary gland, which are overtly manifested by a reduction in milk yield, are also reflected in a reduction of the respiration rate and by a fall in the R.Q. to values near unity. This no doubt means that the synthesis of fat from oxygen-rich compounds is preferentially decreased under these conditions. The effects of adrenalectomy, which also causes a depression of milk yield over and above that ascribable to post-operative anorexia (Cowie & Folley, 1948), could not be quantitatively distinguished from those due to post-operative anorexia. In this respect the enzyme systems responsible for the respiratory metabolism differ from arginase which is believed to play an important role in mammary metabolism in the rat (Folley & Greenbaum, 1947), for the arginase levels of the rat mammary gland were decreased by adrenalectomy but not by partial inanition (Folley & Greenbaum, 1948). The damage to the function of the mammary cells due to these two procedures is associated not only with a decreased respiration, but also by a significant increase in the apparent aerobic glycolysis. Similar effects following weaning will be described in a subsequent paper.

SUMMARY

1. The respiratory metabolism of slices of lactating mammary gland has been studied *in vitro*.

2. Rat mammary tissue shows an appreciable and maintained oxygen uptake in absence of substrate, but this is markedly increased in presence of glucose.

3. Mammary tissue from rats in full lactation gives a $-Q_{O_2}$ (approx. 9 μl./mg. final dry wt./hr.) of the same order as liver but less than that of very active tissues like retina and kidney. The order of activity of tissue from various species is inversely correlated with body size, being as follows: mouse, rat, guinea pig, rabbit, goat, cow.

4. In the presence of glucose the respiratory quotient (R.Q.) of tissue from the mouse, rat, guinea pig and rabbit is well above unity, but that of tissue from ruminants (goat, cow) is less than unity. The results therefore do not support the theory that the lower fatty acids of ruminant milk fat are synthesized from carbohydrate in the udder. The low R.Q. obtained in the absence of substrate in the rat suggests the oxidation of stored fat under these conditions.

5. Of a number of carbohydrates tested, including galactose, fructose and lactose, only mannose and glucose were oxidized by lactating rat mammary gland slices.

6. The aerobic acid production of rat mammary gland slices in presence of glucose is not high, but under anaerobic conditions is quite considerable. Despite the possibility that glycolysis may not account for all the acid produced, it may be concluded that this tissue exhibits a marked Pasteur effect.

7. Slices of mammary gland from rats in which lactation has been depressed by adrenalectomy on the fourth day of lactation or by partial inanition show a lowered Q_{O_2} and R.Q. The damaged cellular function is accompanied by a decrease in the Pasteur effect as evidenced by a significant increase in the apparent aerobic glycolysis.

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Observations on the Mono-amine Oxidase Activity of Placenta and Uterus

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It is known that under aerobic conditions the kidney can oxidize mono-amines such as tyramine or adrenaline by means of a mono-amine oxidase (Blaschko, Richter & Schlossmann, 1937), and it has also been shown that under anaerobic conditions it can decarboxylate tyrosine, dihydroxyphenylalanine and histidine (Werle, 1936; Werle & Hermann, 1937; Holtz, 1937; Holtz, Heise & Spreyer, 1938). More recently a number of experimental findings have suggested the possibility of the excessive production or defective inactivation of pressor amines playing a part in the production of certain types of hypertension (Blaschko, 1942).

The ideas that have been put forward in this connexion have been summarized by Schales (1947), who, in a discussion of the relation of kidney enzymes to essential hypertension, mentions the view that since mono-amine oxidase requires oxygen to inactivate amines 'the restriction of renal circulation in experimental hypertension might make it possible for some pressor amines to escape unoxidized from the kidney', and so play a part in producing peripheral vaso-constriction. Duff, Hamilton & Magner (1939) claim to have produced arteriolar lesions similar to those of malignant hypertension by repeated intravenous injection of tyramine into rabbits. Raska (1943) has reported that the mono-amine oxidase activity of kidney slices from dogs rendered hypertensive by the Goldblatt technique is decreased. His results do not suggest that this decrease is specific, since the rates of residual respiration (i.e. in the absence of added substrate) and of oxidation of DL-alanine, L-dihydroxyphenylalanine (DOPA) and histamine were also diminished. In support of this, Gerbi, Rubinstein & Goldblatt (1940) had earlier found a diminution of the residual oxygen consumption in ischaemic rabbit kidney. Mason, Robinson & Blalock (1940), on the other hand, compared the Q_{O_2} of renal cortical tissue from normal and hypertensive dogs in the presence of glucose and found no significant difference, although they state that preliminary observations showed a striking difference in the rates of residual respiration and of ammonia formation.

Rather more indirect evidence suggesting the possibility of faulty inactivation of the pressor amine formed by decarboxylation of L-DOPA in hypertensive animals has been provided by experiments showing that perfusion of the ischaemic mammalian kidney with DOPA gives rise to the production of a pressor substance having the properties of hydroxytyramine (Bing, 1941). Moreover, the injection of DOPA into cats with experimental renal hypertension causes a marked rise in blood pressure, whereas in normal cats no effect was observed (Bing & Zucker, 1941; Oster & Sorkin, 1942).

In view of the presence in the kidney of this amine-inactivating enzyme, and the suggestions which have been put forward regarding its possible relation to experimental renal hypertension, it was decided to study the possibility of some similar enzymic mechanism being a contributory factor in the development of the hypertension which is a marked and early feature of certain of the toxaemias of pregnancy. In this connexion the finding by Bhagvat, Blaschko & Richter (1939) of a high mono-amine oxidase activity in normal uterus is of interest.

For these reasons a study was undertaken to determine whether an active mono-amine oxidase is present in placenta, since this organ is thought by many workers to play an important part in the pathogenesis of the toxaemia, and also to investigate in greater detail the activity and properties of this enzyme in uterine tissue. Before this work was completed a brief report (Luschinsky & Singher, 1948) appeared on the identification of mono-amine oxidase in human placenta. The present work confirms this observation and describes certain other properties of the enzyme in connexion with the physiology of placental tissue.

METHODS

Estimation of enzymic activity

Oxygen consumption was determined manometrically using the Warburg technique. In all cases, except where stated, the tissue was finely minced with scissors and then dispersed in 0·067 M-phosphate buffer, pH 7·3, in a high-speed homogenizer of the type described by Folley & Watson (1948), so that 2 ml. of the final homogenate corresponded to 200 mg. fresh tissue; where the amount of available tissue was not sufficient to provide a large enough volume for homogenization at this concentration, it was dispersed in the smallest possible volume.

Tyramine hydrochloride (L. Light and Co. Ltd.; recrystallized from water) was used as substrate in 0·01 M solution except where otherwise stated.

O_2 uptake, using 2 ml. of the tissue homogenate, was measured at 38°, and in a gas phase of O_2, over a period of 1 hr., the results being expressed as μl. O_2/g. fresh tissue/hr. Controls for non-enzymic oxidation of the substrate, and for the O_2 consumption of the enzyme preparation in the absence of added substrate, were included in each experiment.

In experiments where it was desired to trap the aldehyde formed during the initial oxidation of the amine in order to prevent further oxidation occurring, semicarbazide hydrochloride was added to the reaction mixture to give a final concentration of 0·02 M.

Ammonia was estimated using the Markham (1942) apparatus.

Materials

Rat, rabbit and guinea-pig placenta. The pregnant uterus was removed immediately after killing the animal by exsanguination. The placentas were then dissected free from the other products of conception, washed with 0·9% NaCl, blotted dry with filter paper, weighed and homogenized.

Human placentas were brought to the laboratory either immediately after delivery or after having been placed immediately in a refrigerator in which they remained for periods up to 10 hr. In most cases they were expelled *per vaginam*, although a few were obtained after Caesarian section. They were prepared by one of two methods:

(1) A small piece, approximately 2 cm. square and extending through the entire thickness of the organ, was excised, mopped free from surface blood, washed with distilled water, weighed and homogenized.

(2) The membranes and larger blood vessels were dissected off, and the remaining tissue squeezed free from as much of the contained blood as possible, after which it was washed with glass-distilled water, mopped with filter paper, weighed, and passed through a Latapie mincer. The mince was then well mixed, and a weighed sample taken for homogenization.

Rat and rabbit uterus. Immediately after killing the animals by exsanguination the uterus was removed, and, in the case of non-pregnant animals, weighed and homogenized. When taken from pregnant animals the whole uterus was removed intact and weighed; if sufficiently developed, the products of conception were then dissected away, and the uterus was mopped free from blood and amniotic fluid with filter paper, washed rapidly in 0·9% NaCl, weighed and homogenized.

RESULTS

Mono-amine oxidase activity of uterus

The presence of an active amine oxidase in dog and sheep uterus was demonstrated by Bhagvat *et al.* (1939). We have extended this finding to the non-pregnant uterus of the rat and rabbit. The results of six typical experiments are shown in Table 1.

Table 1. *Mono-amine oxidase activity of non-pregnant rat and rabbit uterus*

(Activity expressed in μl. O_2/g. fresh tissue/hr.)

Rat	Rabbit
206	505
451	489
222	692
440	500
580	628
322	621

As the object of this work was a study of the enzyme in the pregnant animal, estimations were next made on the rat uterus at various stages of pregnancy. Thirty pregnant rats were used, and

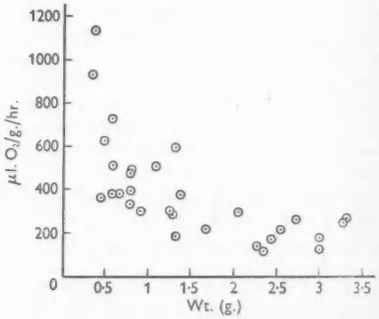

Fig. 1. Relation between mono-amine oxidase activity of pregnant rat uterus (expressed as μl. O_2/g. fresh tissue/hr.) and the weight of the uterus after removal of the products of conception.

a scatter diagram relating the enzyme activities (expressed per g. fresh tissue) to the weight of the uterus after removal of the products of conception is shown in Fig. 1. It is realized that the distribution of such weights affords only an approximate indication of the duration of pregnancy, but owing to the wide variations in the number of foetuses contained in different animals the total weight of uterus, together with products of conception, provides an even less accurate indication.

From the results given in Fig. 1 it will be seen that very high values for enzymic activity (up to 1130 μl. O_2/g./hr.) are obtained early in pregnancy,

these values tending to fall as pregnancy advances to a low level of 100–300 μl. O_2/g./hr. shortly before delivery.

That the relatively high values obtained in the small uterus of early pregnancy are not due merely to the homogenate being necessarily more dilute, with the possibility therefore of an accompanying dilution of some inhibitory factor, is shown by the fact that equally dilute homogenates of non-pregnant uterus do not show these high values. Further, in order to control this point in the pregnant uterus, a direct comparison was made of a uterus using homogenates of three different dilutions; no significant difference between the activities of the three dilutions was observed.

From our present experimental findings it is not possible to interpret this fall in activity per unit weight as pregnancy advances, but it suggests that the enzyme may be localized predominantly in the endometrium, and that the relatively greater mass of myometrium present late in pregnancy may be responsible for the lower activity per unit weight of the whole uterus. With this possibility in view, estimations were carried out on the separated endometrium and myometrium from two human uteruses.

The first was obtained from a normal pregnancy terminated by total hysterectomy at 17 weeks on account of a rapidly growing carcinoma of the breast. The decidua (endometrium in pregnancy), which was thick and plentiful, was separated with a scalpel from the myometrium, and homogenates were made of the two portions of the tissue, the activities of which were measured simultaneously in the usual way; the decidua showed an activity of 663 μl. O_2/g./hr., and the myometrium 97 μl. O_2/g./hr. The second uterus, which was non-pregnant, was removed on account of menorrhagia, and gave values of 432 μl. O_2/g./hr. for the endometrium and 171 μl. O_2/g./hr. for the myometrium.

Two further specimens of human decidua have been obtained by Caesarian section at full term, and have given values of 1125 and 1016 μl. O_2/g./hr.

Mono-amine oxidase activity of placenta

In view of the level of enzymic activity shown by human uterine tissue, human placenta was next investigated. Using small random samples excised from the placenta (method 1, see p. 126), it was found that this tissue was also highly active, values ranging from 338 to 995 μl. O_2/g./hr. being obtained in ten different placentas.

As mentioned above, Raska (1943) has reported a lowering of the mono-amine oxidase level in kidneys from animals rendered hypertensive by the application of the Goldblatt clamp, and since normal human placenta, as well as kidney, appears to be a rich source of the enzyme, it was decided to attempt a comparison of the activity of this enzyme in normal placentas and in those from cases of toxaemia of pregnancy showing hypertension.

The wide variations, however, in the results quoted above obtained on isolated samples of placental tissue led us, when embarking on this comparison, to use homogenates prepared from a portion of a well-mixed mince of the whole placenta, carried out as described above under method 2. Even when using this method it was found that considerable variation exists between the activities of different placentas.

Table 2. *Mono-amine oxidase activities of normal human placentas*

μl. O_2/g. fresh tissue/hr.	mmol. O_2/whole placenta/hr.
665	16·5
510	9·6
660	16·5
660	12·0
925	10·2
535	8·1
406	8·2
705	11·6
825	14·3
808	14·0
555	14·9
825	10·1

The results obtained with twelve different normal placentas at full term are shown in Table 2, where the activities are given both in terms of μl. O_2/g. fresh tissue/hr. and also as mmol. O_2/whole placenta/hr. From these figures, and from the published work on other human tissues (Birkhauser, 1940; Zeller, Stern & Wenk, 1940; Blaschko, 1942; Epps, 1945), it seems that normal human placenta at term must be classed as a tissue relatively rich in mono-amine oxidase.

An active enzyme was also found in rat, rabbit and guinea-pig placenta. In the course of these experiments with animal placentas variations in the level of activity of the mono-amine oxidase were noted in placentas of different ages in different animals. A series of estimations was therefore carried out in rats at various stages of placental development. The results of these experiments are shown in Fig. 2, in which enzymic activities of the placental homogenates from different animals (expressed as μl. O_2/g. fresh tissue/hr.) are plotted against the mean placental weight for each animal.

From the results obtained it will be seen that the level of enzymic activity, expressed per g. of placental tissue, appears to increase as the placenta ages. A linear regression line has been fitted, and has a slope of 55 μl. O_2/g./hr. for each 0·1 g. increase in mean placental weight. The correlation coefficient for the values shown in Fig. 2 is $0{\cdot}76 \pm 0{\cdot}21$. This correlation is in contrast to the results obtained with the whole uterus mentioned above.

If, as has been postulated, any relationship exists between mono-amine oxidase activity in the kidney and experimental renal hypertension, the dependence of enzymic activity on O_2 tension and the relation of this to restriction of the renal circulation becomes important. Further, in view of the postulated association of placental ischaemia with pregnancy toxaemia, this relationship may also be of importance in connexion with the hypertension accompanying this condition. Kohn (1937) has shown that the mono-amine oxidase of pig liver is unusually sensitive to changes in O_2 tension, the rate of oxidation of tyramine being increased by 200–250% by increasing the concentration of O_2 in the gas phase from 20 to 100%. By contrast he showed that a milk xanthine oxidase preparation was almost saturated at 20% O_2.

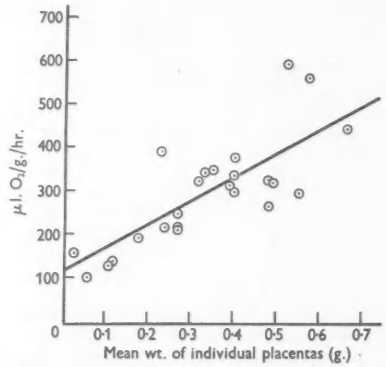

Fig. 2. Relation between mono-amine oxidase activity of rat placenta (expressed as μl. O_2/g. fresh tissue/hr.) and mean weight of the individual placentas from any one animal.

Using homogenates of human placenta we have compared the rates of oxidation of tyramine and of succinate at different oxygen tensions. A final concentration of 0·05 M-sodium succinate was used as substrate in the succinic oxidase determinations.

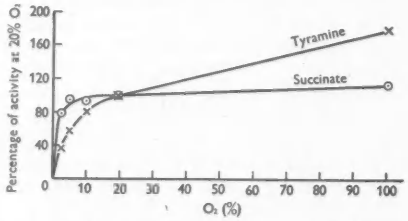

Fig. 3. Effect of O_2 tension on rates of oxidation of tyramine and succinate by human placental homogenates (expressed as percentages of activity at 20% O_2).

The results of these experiments are shown in Fig. 3, in which the enzymic activities are expressed as percentages of the activity at 20% O_2. It will be seen that whereas the rate of oxidation of succinate is only slightly affected by increasing the concentration of oxygen above about 2·5–5%, the rate of oxidation of tyramine increases strikingly as the oxygen tension is increased, the rate in the presence of 2·5% O_2 being less than half that in 10% O_2. In addition to the enzyme in liver, the placental mono-amine oxidase is therefore also highly sensitive to changes in oxygen tension, and any fall in the latter, such as might occur in ischaemic areas of placenta, might therefore be expected to cause a more pronounced fall in the activity of the mono-amine oxidase than in that of certain other enzymes.

Specificity of the placental mono-amine oxidase

The tyramine oxidase first described by Hare (1928) in liver was shown by Blaschko *et al.* (1937) to have a wide range of specificity, oxidizing a number of aliphatic and aromatic mono-amines. In the present work, in addition to tyramine, we have studied the oxidation of 2-phenylethylamine (L. Light and Co.), and tryptamine and DL-adrenaline (British Drug Houses Ltd.), by human placental extracts. These substrates were also used in final concentrations of 0·01 M, the estimations of enzymic activity being carried out as with tyramine. In these experiments, however, semicarbazide was added to prevent the further oxidation of the aldehyde formed by deamination, together with 0·001 M-cyanide to inhibit the oxidation of adrenaline by the cytochrome oxidase system. KCN was also added to the centre cup to prevent loss of cyanide from the main chamber.

The results of these experiments are shown in Table 3. The relative rates of oxidation of tyramine, tryptamine and 2-phenylethylamine by human placenta are of the same order as those found by Blaschko *et al.* (1937) for guinea-pig liver. The value quoted for adrenaline is somewhat higher, but although when using this substrate readings were taken every 10 min., wide variations in its rate of oxidation were found in different experiments.

Table 3. *Relative rates of oxidation of some aromatic amines by placental mono-amine oxidase*

(Extra O_2 uptake due to tyramine/g./hr. taken as 100. Reactions in the presence of 0·02 M-semicarbazide hydrochloride and 0·001 M-KCN.)

Tyramine	100
Tryptamine	77
Adrenaline	128
2-Phenylethylamine	16

A number of experiments were carried out using 0·01 M-histamine dihydrochloride and cadavarine dihydrochloride as substrates, but in each case oxidation by placental homogenates under our conditions was barely measurable in the course of 1 hr.

Ammonia production by placental mono-amine oxidase

The rate of ammonia production has been estimated in a few experiments in order to correlate the extra oxygen uptake with deamination. At the

end of the measurement of oxygen uptake 1 ml. of 25% trichloroacetic acid was added to each bottle. After standing 15 min. the contents of the bottles were centrifuged, and the bottles and the precipitate washed twice with 1 ml. of 5% trichloroacetic acid. The filtrates were then made up to 10 ml. in volumetric flasks, and samples of each taken for ammonia estimation.

In each of these experiments the oxygen consumption in the presence of semicarbazide was also estimated, in order to determine the proportion of total oxygen uptake by the placental enzyme which was due to oxidation of the aldehyde formed. The results of these experiments are shown in Table 4.

Table 4. *Ratio of atoms oxygen utilized/moles ammonia produced, and ratio of oxygen utilized in the presence and absence of semicarbazide, during oxidation of tyramine by the placental enzyme*

O_2 uptake (μl. O_2/g./hr.)			Atoms O/moles NH_3		
Without semicarbazide (*a*)	With semicarbazide (*b*)	NH_3 produced (μM/g./hr.)	Without semicarbazide	With semicarbazide	Ratio of O_2 uptakes (*a*)/(*b*)
672	521	43·9	1·37	1·06	1·29
601	506	48·6	1·10	0·92	1·19
680	595	52·4	1·16	1·01	1·16
890	735	55·7	1·42	1·17	1·21

In the absence of semicarbazide a mean O:NH_3 ratio of 1·26 was obtained; when, however, the figures for the oxygen uptake in the presence of semicarbazide are used, the ratios in these four experiments become 1·06, 0·92, 1·01 and 1·17 respectively.

DISCUSSION

The findings reported in this paper establish the presence of an active mono-amine oxidase in placental extracts, confirming the recent observations of Luschinsky & Singher (1948). From a study of its action on different amines it has been shown that this enzyme, like the mono-amine oxidase present in liver, kidney and elsewhere, can oxidize a number of aromatic mono-amines, but is inactive against di-amines such as histamine or cadaverine.

In human placenta at term, this enzyme is present in relatively large amounts, and in rat placenta its activity appears to increase as the placenta matures. From the experiments which we have made it is impossible to do more than speculate on the possible significance of this enzyme in placenta, but the fact that in rat placenta its activity appears to increase as the placenta matures suggests that it may exercise some function of increasing significance as pregnancy advances, and the foetus or foetuses increase in size and differentiation. In this connexion it should be noted that Epps (1945) has shown that the kidney cortex and medulla of the newborn child are deficient in mono-amine oxidase, the activity gradually increasing until the child is about 3 months old, when maximum activity appears to be reached. In a study of the DOPA-decarboxylase activity of various human and animal tissues Page (1945), using a biological method for estimating the hydroxytyramine formed, was unable to detect the presence of this enzyme in human placenta. As far as we are aware, however, the decarboxylation of other amino-acids by placental or foetal tissue has not so far been studied.

The demonstration by Kohn (1937) that the activity of the mono-amine oxidase of liver is determined by the oxygen tension has been shown to apply also to the enzyme in placenta. In the absence of any exact information as to the oxygen tensions to which the enzyme would normally be exposed under *in vivo* conditions, it is clearly impossible to interpret this property of amine oxidase in any physiological sense. It does suggest, however, that any lowering of the local oxygen tension, such as might be expected to occur in the placenta, more particularly towards the end of pregnancy, might result in an inhibition of the activity of this enzyme to a greater extent than that of the succinic oxidase system, and possibly also of other systems involving the cytochrome-cytochrome oxidase mechanism.

The high values of the mono-amine oxidase activity of the rat uterus early in pregnancy together with the apparent fall in these values, when expressed on a weight basis as pregnancy advances, suggests that the enzyme may be chiefly localized in the endometrium, and this supposition has been confirmed by examination of the separate endometrium and myometrium from human uterus. The low value found in the myometrium would indicate that this muscle is similar to the other muscular organs examined by Bhagvat *et al.* (1939), the high value of whole uterus being due mainly to an endometrial enzyme.

Experiments are now in progress in the hope of obtaining information concerning the physiological significance of this enzyme in placenta, and to ascertain whether any abnormalities of its function are detectable in the toxaemias of pregnancy.

SUMMARY

1. The presence of an active mono-amine oxidase in uterus has been confirmed. Evidence is presented indicating that in the human uterus, and possibly also in the rat uterus, it is mainly localized in the endometrium.

2. Human, rat, rabbit and guinea-pig placentas have also been found to be potent sources of the enzyme.

3. The level of enzymic activity in rat placenta (expressed per g. of tissue) appears to increase as the placenta matures *in utero*.

4. The activity of the placental enzyme, like that of the enzyme in liver, is proportional to the oxygen tension of the medium in which it acts, a fall of oxygen tension causing a marked fall in rate of enzyme action.

We would like to acknowledge the help given to us in the collection of human material by Mr J. B. Blaikley, F.R.C.S., F.R.C.O.G., and the staff of the Department of Obstetrics and Gynaecology, Guy's Hospital Medical School. Our thanks are also due to Mr R. F. Adams and Miss E. Smalley for technical assistance.

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Effect of Sulphydryl-combining Compounds on the Activity of the Succinic Oxidase System

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(*Received* 22 *November* 1948)

Thunberg (1916) was the first to suggest that the tissue catalyst responsible for the oxidation of succinate contained a sulphydryl group, but his evidence, viz. that colloidal sulphur inhibited the enzyme, was not very convincing. There was, in fact, better evidence available to Thunberg, since Battelli & Stern (1910) had already shown that arsenite inhibited this enzyme, and Ehrlich (1909) had suggested that the toxic action of arsenicals on tissues might be due to their combination with sulphydryl groups. This suggestion of Ehrlich has recently received convincing experimental support by the work of Peters and his colleagues (Peters, Stocken & Thompson, 1945).

Hopkins and his co-workers (Hopkins & Morgan, 1938; Hopkins, Morgan & Lutwak-Mann, 1938) were the first to demonstrate the existence and the importance of SH groups in succinic dehydrogenase. These workers showed that succinic dehydrogenase was completely inhibited by incubation with a high concentration of oxidized glutathione (GSSG), and that the activity was completely restored by incubating the inhibited enzyme with reduced glutathione (GSH) under anaerobic conditions. They suggested that GSSG acted by oxidizing the SH groups of succinic dehydrogenase to disulphide (S.S) groups and that this reaction was reversible.

$$GSSG + 2EnSH \rightleftharpoons En.S.S.En + 2GSH.$$

Since the publication of the papers of Hopkins and his co-workers, many studies of the action of other SH-combining compounds on succinic oxidase

have appeared (e.g. Morgan & Friedmann, 1938; Potter & Dubois, 1943; Barron & Singer, 1945; Barron & Kalnitsky, 1947; Mackworth, 1948). Five different types of compounds have been used, viz. alkylating agents, oxidizing agents, arsenicals, mercaptide-forming agents and maleic acid.

Despite these many studies of the action of SH agents, an examination of the literature revealed that certain points required further investigation. The most important of these was the reversibility of the inhibition, concerning which the literature presents a confusing picture. Hopkins *et al.* (1938) showed that treatment with GSH completely reactivated the succinic oxidase system in washed minced muscle after it had been inactivated by GSSG. Potter & Dubois (1943), on the other hand, using a tissue homogenate as the source of the enzyme, were unable, by means of GSH, to reverse the inhibition caused by various oxidizing agents. They explained the disagreement between their findings and those of Hopkins *et al.* by pointing out an important difference in the experimental procedures. Hopkins *et al.* treated the inactivated enzyme with GSH under anaerobic conditions, and, after this treatment, removed the excess GSH by filtering and washing the muscle pulp. Since this procedure is not possible when dispersions are used as the source of the enzyme, Potter & Dubois did not remove the GSH before measuring the succinic oxidase activity. GSH is fairly rapidly oxidized when added to such tissue preparations in the presence of air (especially if excess cytochrome *c* is added), and so cannot be an effective reactivating agent. In fact, Potter & Dubois (1943) and Ames & Elvehjem (1944) found that GSH and cysteine actually inhibited the succinic oxidase activities of tissue preparations. The presence of the thiol in the reaction mixture during the measurement of the activity of the enzyme introduces a further difficulty, viz. that the absorption of oxygen causes an error in the determination of the succinic oxidase activity. In opposition to the findings of Potter & Dubois, Barron & Singer (1945) claim that the succinic oxidase activity of a muscle dispersion, previously inhibited to the extent of 80% by treatment with benzoquinone or *p*-carboxyphenylarsine oxide, was completely restored by treatment with GSH (50 times the concentration of the inhibitor). These authors state that the 'reactivation produced by glutathione was instantaneous and in no way connected with its oxidation by metal catalysts in the enzyme, since the enzyme suspension showed no appreciable oxygen uptake on addition of glutathione without succinate during the course of the experiment (20 min.)'. Barron, Miller, Bartlett, Meyer & Singer (1947) later showed that the dithiol 2:3-dimercaptopropanol (BAL) was more effective than GSH in reversing the inhibition by arsenicals.

A further point requiring study is the component of the succinic oxidase system which is attacked by the inhibitor. Some authors, although using the rate of oxygen uptake in the presence of succinate as the measure of the enzyme activity, speak of the enzyme concerned in this oxidation as succinic dehydrogenase. Actually, an enzyme complex, of which succinic dehydrogenase is only one component, is necessary to catalyse the aerobic oxidation of succinate.

An additional reason for these experiments was to study the action of SH-combining compounds on the succinic oxidase system in Keilin & Hartree's heart-muscle preparation, which, although it is the most active preparation of this enzyme system, has not been used by previous investigators studying these inhibitors.

Three types of inhibitors were used, viz. (1) oxidizing agents: *o*-iodosobenzoate and GSSG; (2) *p*-aminophenylarsenoxide; (3) *p*-chloromercuribenzoate. Alkylating agents were not studied, since this inhibition is not easily reversible. A preliminary account of some of these findings has already appeared (Slater, 1948).

METHODS

Enzyme preparation. Keilin & Hartree's (1947) heart-muscle preparation was used. The methods of preparing the enzymes and determining the activities have already been described (Slater, 1949*a*, *b*).

Glutathione was prepared by the methods of Hopkins (1929) and Pirie (1930). Aqueous solutions were neutralized immediately before use. GSSG was prepared by shaking a neutralized solution of GSH in a manometric flask until no more O_2 was absorbed.

BAL was kindly provided by Prof. R. H. S. Thompson. Aqueous solutions were prepared immediately before use.

o-Iodosobenzoic acid was prepared by the oxidation of *o*-iodobenzoic acid with fuming HNO_3, according to the method of Meyer & Wachter (1892). It was 90% pure according to the amount of I_2 liberated from acidified KI.

p-Chloromercuribenzoate was kindly supplied by Dr S. V. Perry.

Procedure. The general procedure adopted was to treat a concentrated enzyme preparation with the inhibitor for a definite length of time and then rapidly to dilute about 50-fold before measuring the enzyme activity. Except in the case of *p*-chloromercuribenzoate, this rapid dilution practically stopped the action of the inhibitor on the enzyme. Unless otherwise stated, 1 ml. of Keilin & Hartree's (1947) heart-muscle preparation was treated with the inhibitor solution; after standing for the required time, the solution was diluted to 5 ml. with 0·18M-phosphate buffer, pH 7·3, and 0·2 ml. samples were pipetted into the manometer flasks and immediately diluted to 3·1 ml. with phosphate buffer and the other solutions necessary for the measurement of the enzyme activity, which was carried out as soon as possible.

This method is valid only if dilution of the enzyme does not cause any reversal of the inhibitions. This was found to be the case.

EXPERIMENTAL

A. *Effect of sulphydryl-combining agents on the succinic oxidase system*

(1) *Rate of inhibition.* The effect of the time of contact of the inhibitor with the enzyme at 37° on the degree of inhibition is shown for four inhibitors in Fig. 1. The figures at zero time were obtained by adding the inhibitor and immediately diluting. The small inhibitions found with *o*-iodosobenzoate and *p*-aminophenylarsenoxide at zero time probably occurred during this dilution or after the dilution.

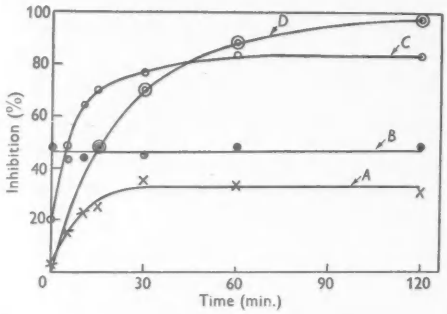


Fig. 1. Rate of inactivation, at 37°, of succinic oxidase system by 0·2 μequiv. *o*-iodosobenzoate per ml. heart-muscle preparation (curve *A*); 0·5 μequiv. *p*-chloromercuribenzoate per ml. heart-muscle preparation (curve *B*); 0·2 μequiv. *p*-aminophenylarsenoxide per ml. heart-muscle preparation (curve *C*); 20 μequiv. GSSG per ml. heart-muscle preparation (curve *D*).

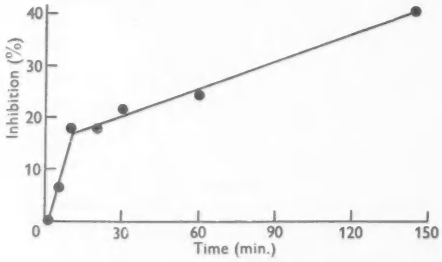


Fig. 2. Rate of inactivation, at 16°, of the succinic oxidase system by 2 μequiv. *o*-iodosobenzoate per ml. heart-muscle preparation.

Inhibition by *p*-aminophenylarsenoxide and by *o*-iodosobenzoate, and particularly by GSSG (even in large excess) was a slow process; maximum inhibition was found with the arsenical and *o*-iodosobenzoate after 1 hr. The reaction between the enzyme and *p*-chloromercuribenzoate was much more rapid. It cannot be stated from Fig. 1 whether this inhibition occurred practically instantaneously before or after dilution. It can, however, be definitely concluded that, under comparable conditions, the rate of inhibition of the enzyme by *p*-chloromercuribenzoate is much greater than that by the other inhibitors tried. The succinic dehydrogenase, which, as is shown below, is less susceptible to the mercurial than is the complete succinic oxidase system, was just as rapidly inactivated by the mercurial.

In Fig. 2, the rate of inhibition of the enzyme by *o*-iodosobenzoate at 16° is shown. It appears from the shape of the curve that the inhibition at this temperature was due to two reactions; an initial fairly rapid reaction which was complete after 10 min., followed by a slower reaction, which was not complete after 2 hr.

(2) *Effect of amount of inhibitor.* The effect of different amounts of *o*-iodosobenzoate, *p*-aminophenylarsenoxide and *p*-chloromercuribenzoate on the succinic oxidase activity of the same heart-muscle preparation is shown in Fig. 3. Sufficient time was allowed for complete reaction between the

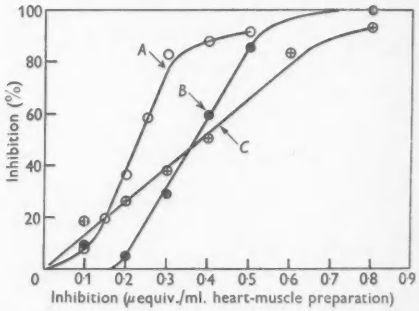


Fig. 3. Effect of different amounts of *p*-aminophenylarsenoxide (curve *A*), *o*-iodosobenzoate (curve *B*), and *p*-chloromercuribenzoate (curve *C*) on the succinic oxidase system. *p*-Amino-phenylarsenoxide and *o*-iodosobenzoate: reaction carried out at 37° for 60 min.; *p*-chloromercuribenzoate: 37° for 15 min.

inhibitor and the enzyme. A considerable amount of the arsenical or *o*-iodosobenzoate was required before appreciable inhibition occurred; the inhibition increased markedly with further small increases in the amount of inhibitor until nearly complete inactivation was obtained. The unusual shape of the curves is probably due to the presence, in the heart-muscle preparation, of substances (possibly denatured proteins, which are known to contain many reactive SH groups) which combine preferentially with these two inhibitors and thus protect the enzyme from inactivation by small concentrations of the inhibitor. The heart-muscle preparation often became less susceptible to SH-combining reagents (particularly to *o*-iodosobenzoate) after standing for a few days,

probably due to an increase in the amount of denatured proteins.

These substances in the heart-muscle preparation which protected the enzyme from small amounts of arsenical and oxidizing agent were quite ineffective in protecting against the same amount of the mercurial. On the other hand, the enzyme was more susceptible to relatively larger amounts of the arsenical and *o*-iodosobenzoate than to the corresponding amount of mercurial. These findings indicate that the mercurial was much less selective in its action than the other two compounds. The arsenical was the most selective agent of the three.

(3) *Component of the succinic oxidase system inhibited by the sulphydryl reagents.* In Fig. 4 the effect of different amounts of *p*-aminophenylarsenoxide on the activity of both the complete succinic oxidase system and on the succinic dehydrogenase

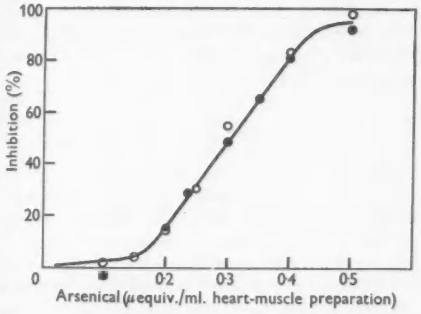


Fig. 4. Effect of different amounts of *p*-aminophenylarsenoxide on the succinic oxidase system ○, and on succinic dehydrogenase ●. Reaction carried out at 37° for 60 min.

portion is shown. The fact that the two curves coincide means that the component of the succinic oxidase system which is limiting the activity of the system after treatment with the arsenical is the succinic dehydrogenase, and that the primary action of the arsenical is on the dehydrogenase.

The oxidizing agents, *o*-iodosobenzoate and GSSG, on the other hand, have a greater effect on the complete system than on the succinic dehydrogenase (Figs. 5 and 6). Cupric chloride, which may act primarily as an oxidizing agent, also has a much stronger action on the complete system than on the dehydrogenase. Hellerman, Perkins & Clark (1933) and Mapson (1946) have shown that Cu^{++} does not rapidly inactivate urease, whereas Cu^{+} is strongly inhibitory. Hopkins (1929) found that GSH reacted with Cu^{++} by first reducing it to Cu^{+}, which then combined with unreacted GSH. It is possible that the same type of reaction occurs with the succinic oxidase system; the first action of the Cu^{++} may be an oxidative reaction on some component of the system, leading to inactivation of the succinic oxidase system, and this is followed by combination of the Cu^{+} formed with the SH groups of the succinic dehydrogenase.

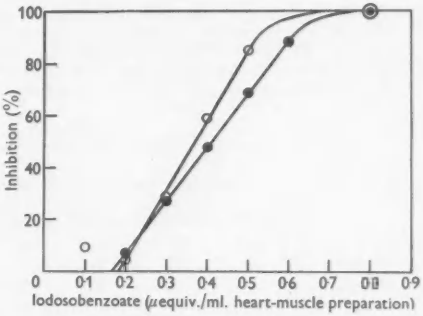


Fig. 5. Effect of different amounts of iodosobenzoate on the activity of succinic oxidase ○, and of the succinic dehydrogenase ●. Reaction carried out at 37° for 60 min.

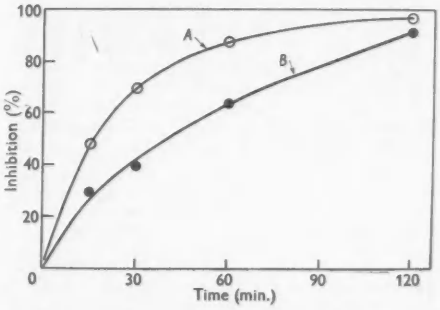


Fig. 6. Rate of inactivation of succinic oxidase (○, curve *A*) and succinic dehydrogenase (●, curve *B*) by GSSG (20 μequiv./ml. heart-muscle preparation). Temp. 37°.

Since Hellerman *et al.* (1933) found that Cu^{++} catalysed the aerobic destruction of urease, it was of interest to determine whether it had the same effect on the succinic oxidase system. The figures in Table 1 show that this is not the case; Cu^{++} had the same inhibitory effect whether it was tested in the presence or in the absence of air.

The effect of different amounts of *p*-chloromercuribenzoate on the succinic oxidase and succinic dehydrogenase activities is shown in Fig. 7. The Thunberg method of measuring succinic dehydrogenase activity was employed, since potassium cyanide, which is used in the manometric method to inhibit cytochrome oxidase, reverses the inhibition of the dehydrogenase (see p. 137). The mercurial, like the oxidizing agents but unlike the arsenical, inhibits the complete succinic oxidase system more than the dehydrogenase.

Table 1. *Effect of cupric ions on the succinic oxidase system and on succinic dehydrogenase*

(Heart-muscle preparation (1 ml.) + 0·4 ml. $CuCl_2$ were either shaken in a manometer flask in the presence of air (aerobic) or kept *in vacuo* ($CuCl_2$ added after evacuation) in a Thunberg tube (anaerobic) for 15 min. at 37°. Concentration of Cu^{++}: $7·2 \times 10^{-5}$ M. 3·6 ml. 0·18 M-phosphate buffer were then added and 0·2 ml. samples taken for measurement of enzyme activities. Control without Cu^{++} treated in same way.)

	Succinic oxidase activity (μl. O_2/10 min.)		Succinic dehydrogenase activity (μl. O_2/10 min.)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Control	112	110	41	40
In presence of Cu^{++}	46	44	29	27
Inhibition (%)	59	60	29	34

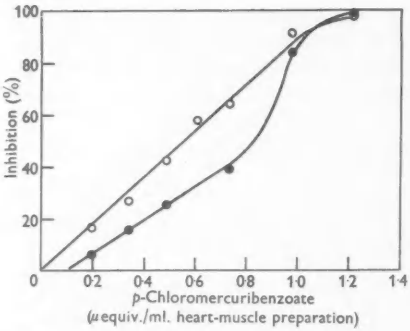


Fig. 7. Effect of different amounts of *p*-chloromercuribenzoate on the activity of succinic oxidase ○, and of the succinic dehydrogenase ●. Reaction carried out at 37° for 15 min.

The effect of the arsenical, the mercurial and *o*-iodosobenzoate on the cytochrome oxidase activity is shown in Table 2. Only the mercurial had an appreciable effect on the cytochrome oxidase. The insensitivity of cytochrome oxidase to arsenicals and oxidizing agents is in agreement with the findings of Hopkins *et al.* (1938) with GSSG and Barron & Singer (1945) with arsenicals.

Table 2. *Effect of sulphydryl-combining agents on the cytochrome oxidase of heart-muscle preparation*

(Heart-muscle preparation treated with inhibitors for 1 hr. at 37°. General procedure: see Methods. Note that the cytochrome oxidase activity measured was the true activity at infinite cytochrome *c* concentration, measured by the method of Slater (1949*b*).)

Inhibitor	Inhibition (%)
p-Chloromercuribenzoate (1·25 μequiv.)	29
o-Iodosobenzoate (1·0 μequiv.)	5
p-Aminophenylarsenoxide (0·5 μequiv.)	5

B. *Reactivation*

The inhibition produced by treatment with *p*-aminophenylarsenoxide was not reversed when the concentration of the inhibitor was reduced by diluting the reaction mixture (Table 3), nor even by precipitating the enzyme with acid and washing away the unreacted inhibitor. Similarly, the inhibition by *o*-iodosobenzoate was not reversed by the latter treatment (Table 4). After treatment with *p*-chloromercuribenzoate also, the enzyme was not reactivated by dilution.

Table 3. *Effect of dilution of heart-muscle preparation, after treatment with* p*-aminophenylarsenoxide, on the degree of inhibition*

(*Inhibited:* 1 ml. heart-muscle preparation + 0·5 ml. 0·0004 N-*p*-aminophenylarsenoxide kept at 37° for 1 hr.; 3·5 ml. 0·18 M-phosphate buffer added and different volumes pipetted into manometer flasks for measurement of succinic oxidase activity in the usual manner. *Control:* 1 ml. heart-muscle preparation + 0·5 ml. water treated similarly.)

Final dilution of heart-muscle preparation in manometer flasks	O_2 uptake (μl./10 min.)		Inhibition (%)
	Control	Inhibited	
1 : 82	61	35	43
1 : 41	118	69	42
1 : 24	187	112	40
1 : 16	231	141	39

Table 4. *Inhibition of succinic oxidase by o-iodosobenzoate; attempt to reverse inhibition by washing enzyme with water*

(A, 1 ml. heart-muscle preparation treated with *o*-iodosobenzoate (total volume = 1·1 ml.) for 30 min. at 16°; 3·9 ml. 0·15 M-phosphate buffer added. B, 1 ml. heart-muscle preparation treated with *o*-iodosobenzoate as A. Cooled to 0° and 4 ml. 0·01 N-acetic acid added, centrifuged, supernatant fluid removed, residue stirred with 2 ml. water, centrifuged and residue suspended in 5 ml. 0·15 M-phosphate buffer. 0·2 ml. samples taken for measurement of enzyme activity. Control tests showed that untreated enzyme could be precipitated with acid, washed and resuspended in phosphate buffer in this manner without loss of activity.)

o-Iodosobenzoate (μequiv./ml. heart-muscle preparation)	Inhibition (%)	
	A	B
2	71	78
4	85	83

The enzyme can, however, be reactivated by treatment with SH compounds. It was found, in agreement with Potter & Dubois (1943) and Ames & Elvehjem (1944) and in disagreement with Barron & Singer (1945), that complete reactivation of the succinic oxidase system could not be effected by adding the thiol to the enzyme preparation in the presence of air, since the thiol was oxidized by the

enzyme preparation and this oxidation was accompanied by inactivation of the succinic oxidase system. Ames & Elvehjem (1944) believed that this inhibition, caused by cysteine and GSH, was due to the action of cystine and GSSG formed by oxidation of the thiol compounds. It was found in the present study, however, that this could not be the complete explanation since the inhibition caused by GSH is much greater than that resulting from treatment with the equivalent concentration of GSSG. This observation was the starting point of the investigation of reducing agents on the succinic oxidase system which is described elsewhere (Slater, 1949*c*). It was found that GSH had no effect on the enzyme under completely anaerobic conditions, and that, even in the presence of air, the succinic dehydrogenase was much less susceptible than the complete succinic oxidase system to the thiol. Thus, in order to study the reversal of the inhibition, it is necessary to use Hopkins & Morgan's (1938) procedure of treating the enzyme with the thiol under anaerobic conditions. When using dispersions, it is difficult to remove the thiol from the enzyme quickly after the treatment before the measurement of the enzyme activity. This difficulty was overcome by treating a high concentration of enzyme with the thiol under anaerobic conditions and quickly diluting the suspension about 50 times before measuring the enzyme activity. The final very low concentration of the thiol was then without appreciable effect on the enzyme. In some experiments, such a high concentration of thiol was necessary to reactivate the enzyme that the thiol concentration, even after this 50-fold dilution, was appreciable and caused a small inactivation of the enzyme. In these experiments, the extent of the inactivation was determined in a separate control experiment and taken into account when calculating the degree of reactivation of the enzyme (see Fig. 8).

In the presence of air, the relative effectiveness of different thiols in reversing the inhibition caused by SH-combining reagents will depend, not only on the relative affinity of the thiol and the enzyme for the inhibitor, but also on the rate of oxidation of the thiol. The latter factor must be taken into account when considering the finding of Barron & Kalnitsky (1947) that several dithiols were more effective than BAL in reversing the inhibition of the succinic oxidase system caused by various heavy metals, since these authors worked under aerobic conditions and Barron, Miller & Kalnitsky (1947) had previously shown that these other dithiols were oxidized more slowly than BAL.

(1) p-*Aminophenylarsenoxide*

(*a*) *Rate of reactivation.* The heart-muscle preparation, after treatment with 0·5 μequiv. of *p*-aminophenylarsenoxide per ml. of heart-muscle preparation for 30 min. at 37°, was reactivated by treatment with BAL for various times before dilution. The results, which are given in Fig. 8, show that the reactivation was quite rapid. It should be noted that the times of contact with BAL refer only to the time before dilution. It seems likely, from the shape of the curve, that some reaction took place either during or after dilution and the curve should not be taken to represent the true rate of reactivation. It can be seen, however, by comparison with Fig. 1, obtained under the same conditions, that the rate of reactivation was considerably greater than the rate of inactivation.

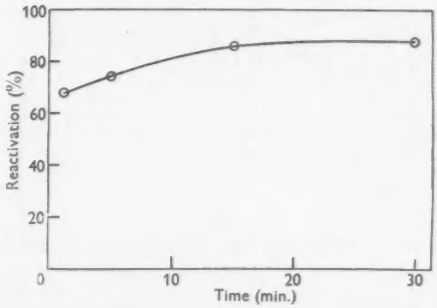


Fig. 8. Rate of reactivation of succinic dehydrogenase, after inhibition by *p*-aminophenylarsenoxide, by treatment with BAL under anaerobic conditions; equiv. BAL/equiv. *p*-aminophenylarsenoxide = 3·76. Mixture *A*, 12 ml. heart-muscle preparation + 6 ml. water kept at 37° for 30 min.; 2 ml. *A* diluted with 4·4 ml. 0·18 M-phosphate buffer, and succinic dehydrogenase activity of 0·2 ml. of diluted solution determined; value *a*. Mixture *B*, 12 ml. heart-muscle preparation + 6 ml. 0·0005 M-*p*-aminophenylarsenoxide kept at 37° for 30 min.; 2 ml. *B* diluted with 4·4 ml. 0·18 M-phosphate buffer, and succinic dehydrogenase activity of 0·2 ml. diluted solution determined; value *b*. Samples of *A* (2 ml.) placed in Thunberg tubes, containing 0·25 ml. 0·005 M-BAL in hollow stoppers. Evacuated, contents of Thunberg tubes mixed and reacted for various times before rapidly diluting with 4·4 ml. 0·18 M-phosphate buffer; 0·2 ml. of diluted solutions taken immediately for measurement of succinic dehydrogenase activity; value *c*. Samples of *B* (2 ml.) treated in exactly the same manner; value *d*. Percentage inactivation = 100 $(a-b)/a = 88$; percentage reactivation = 100 $(ad/c-b)/(a-b)$.

(*b*) *Reactivation by different concentrations of BAL and GSH.* The effect of different concentrations of BAL on the degree of reactivation is shown in Fig. 9, which includes measurement both of succinic dehydrogenase and of the complete succinic oxidase system. The succinic dehydrogenase and the complete system were reactivated to about the same extent. Under the conditions of this experiment, the reactivation was not quite complete, amounting to

about 80% of full reactivation. This may be because, during the necessary manipulations, the enzyme-arsenical compound was allowed to stand for over 1 hr. before the addition of the BAL. Barron & Singer (1945) found that complete reactivation of the enzyme inhibited by *p*-chloromercuribenzoate occurred only when the thiol (GSH) was added soon after the mercurial and suggested that the enzyme whose SH groups are combined is readily denatured on standing. The most interesting feature of Fig. 9 is the small amount of BAL required to produce the maximum reactivation, viz. about twice the amount of BAL equivalent to the arsenical added; BAL exactly equivalent to the arsenical produced 80% of the maximum reactivation obtained. When a similar experiment was carried out in an open test tube, BAL equivalent to 2·5 times the amount of arsenical reactivated to the extent of only 36%, even allowing for the inactivating effect of BAL itself. These findings may be compared with those of Barron, Miller, Bartlett, Meyer & Singer (1947), who found that BAL equivalent to 2·5 times the concentration of lewisite reactivated the succinic oxidase system by 83%, while double this amount completely reactivated the system. The amount of BAL required to reverse the inhibition of the succinic dehydrogenase by *p*-aminophenylarsenoxide is considerably less than that found by Stocken, Thompson & Whittaker (1947) to be necessary to reactivate the pyruvic oxidase system after the action of various therapeutic arsenicals.

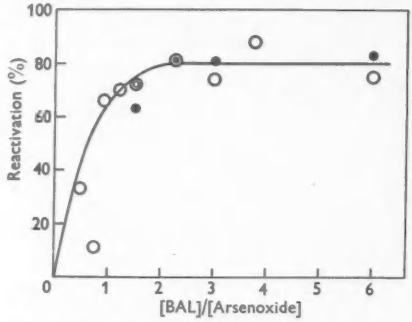


Fig. 9. Reactivation of succinic dehydrogenase and succinic oxidase, after treatment with *p*-aminophenylarsenoxide (0·5 μequiv./ml. heart-muscle preparation), by treatment with various concentrations of BAL under anaerobic conditions. ●, succinic oxidase; ○, succinic dehydrogenase. Procedure as Fig. 8; reactivation time, 30 min.; inactivation, 85%.

GSH was much less effective than BAL in reversing the inhibition; thus, GSH equivalent to 1·5 times the concentration of arsenical produced no measurable reactivation, while 7·5 times the equivalent concentration caused 36% reactivation. This result is similar to that obtained by Barron, Miller, Bartlett, Meyer & Singer (1947), who found that GSH equal to 5 times the lewisite used reversed the inhibition by 23%.

(2) o-*Iodosobenzoate*

The experiments reported in Table 5 show that much greater amounts of thiol are required to reverse the inhibition caused by *o*-iodosobenzoate. BAL equivalent to 10 times the amount of oxidizing agent reactivated the succinic dehydrogenase by 61%, while BAL equivalent to 50 times the amount of iodosobenzoate brought about nearly complete reactivation. GSH was rather less effective than

Table 5. *Reactivation of succinic dehydrogenase and of succinic oxidase after inhibition by o-iodosobenzoate*

(Exp. 1: 5 ml. heart-muscle preparation treated with 5 ml. 0·001 M-*o*-iodosobenzoate for 30 min. at 37°; 2 ml. samples pipetted into Thunberg tubes containing BAL or GSH in hollow stoppers. Contents mixed after evacuation of tubes. After 30 min. at 37°, tube opened, contents quickly diluted to 5 ml. with phosphate buffer, 0·2 ml. samples pipetted into manometer flasks and quickly diluted with buffer. Exp. 2: Thunberg tubes contained 1 ml. heart-muscle preparation and 0·3 ml. 0·001 M-iodosobenzoate in main compartments and BAL or GSH in hollow stoppers. Evacuated, allowed to react for 30 min. at 37°, then mixed and allowed to stand a further 30 min. at 37°. Subsequent treatment as in Exp. 1. Percentage reactivation calculated as in Fig. 8.)

μequiv. *o*-iodosobenzoate/ml. heart-muscle preparation	Inhibition (%)		Reactivating agent	Ratio of BAL or GSH (equiv.): iodosobenzoate (equiv.)	Reactivation (%)	
	Succinic oxidase	Succinic dehydrogenase			Succinic oxidase	Succinic dehydrogenase
			Exp. 1			
2·0	—	57	BAL	1·5	—	12
			BAL	5	—	29
			GSH	1·5	—	0
			Exp. 2			
0·6	96	89	BAL	10·5	26	61
			BAL	50	54	92
			GSH	50	32	70

BAL. The inhibition of the complete system was much less readily reversible than that of the succinic dehydrogenase.

(3) *Oxidized glutathione*

Owing to the necessity of using very high concentrations of GSSG to inhibit the enzyme, it was not possible to use a large excess of thiol to reactivate the enzyme. In the experiment shown in full in Table 6, BAL, equivalent to twice the concentration

Table 6. *Reactivation of succinic dehydrogenase and succinic oxidase after treatment with oxidized glutathione*

(A, 1 ml. heart-muscle preparation kept at 37° for 1 hr., diluted to 5 ml. and succinic oxidase and succinic dehydrogenase determined on 0·2 ml. samples. B, 1 ml. heart-muscle preparation + 0·2 ml. 0·05 M-GSSG (prepared by aerobic oxidation of neutralized 0·1 M-GSH) kept at 37° for 1 hr. Then as A. C, 1 ml. heart-muscle preparation in main tube and 0·2 ml. 0·1 M-BAL in hollow stopper. Contents mixed after evacuation and kept at 37° for 30 min. Then as A. D, 1 ml. heart-muscle preparation in main tube and 0·2 ml. 0·1 M-BAL in hollow stopper. 0·2 ml. 0·05 M-GSSG added to heart-muscle preparation; kept at 37° for 1 hr. Then evacuated, contents of hollow stopper mixed with those of main tube and kept at 37° for 30 min. Then as A.)

Exp.	Succinic oxidase (μl. O_2/10 min.)	Succinic dehydrogenase (μl. O_2/20 min.)
A	101·7	62
B	46·5	41·1
C	78·9	55·2
D	16·6	53·6
Inhibition by GSSG (%)		
$\frac{A-B}{A}\times 100$	56	36
Reactivation by BAL (%)		
$\frac{AD/C-B}{A-B}\times 100$	−45	+91

of GSSG used to inhibit the enzyme, completely reactivated the succinic dehydrogenase, but did not reactivate the complete succinic oxidase system. In fact, the inhibition of the latter increased during the treatment with BAL. It is impossible to compare the relative ease of reversibility after treatment with *o*-iodosobenzoate and GSSG, since the amount of the latter used was so much greater than that of the former that the concentration of BAL present in the experiment with GSSG was greater than in the experiment with *o*-iodosobenzoate, although the relative concentrations of reactivating agent to inhibitor were much greater in the latter experiment. Further information on the reversibility of the enzyme system after treatment with GSSG could probably be obtained if the enzyme was sedimented by high speed centrifugation and washed free from GSSG before adding the reactivating agent.

Hopkins *et al.* (1938) treated the washed muscle with GSSG under anaerobic conditions. It was found in the present study that the inhibitions of both the complete succinic oxidase system and of succinic dehydrogenase and the reactivations by BAL were the same, whether the enzyme was treated with the GSSG in the presence or in the absence of air.

Inhibition by GSSG was not reversed by 0·01 M-cyanide, the concentration used in the measurement of the succinic dehydrogenase by the manometric method, with methylene blue as carrier. This was shown by the fact that the degree of inhibition was the same whether the succinic dehydrogenase activity was measured by this manometric or by the Thunberg method, in which cyanide is not used.

(4) p-*Chloromercuribenzoate*

Heart-muscle preparation, treated with sufficient *p*-chloromercuribenzoate to inhibit completely the oxidation of succinate by methylene blue in the absence of potassium cyanide, was reactivated by 80% by the addition of 0·01 M-cyanide. Strangely, the inhibition was not reversed if cyanide was added to the Barcroft flask after the succinate.

After complete inhibition by 1·5 μequiv. of *p*-chloromercuribenzoate per ml. of enzyme preparation, the complete succinic oxidase system was reactivated by 31% with an amount of GSH equivalent to the mercurial used and by 65% with 5 times as much GSH. The succinic dehydrogenase was reactivated to the same extent as the complete system. The enzyme was also readily reactivated (73%) by adding 0·13% denatured globin to the manometric flask in which the enzymic activity was measured.* In marked contrast, however, the enzyme was not in the slightest degree reactivated by BAL in an amount equivalent to 5 times the amount of mercurial used.

C. *Spectroscopic observations*

A normal cytochrome spectrum was obtained when sodium dithionite ($Na_2S_2O_4$) was added to heart-muscle preparation treated with sufficient *p*-aminophenylarsenoxide, *o*-iodosobenzoate or *p*-chloromercuribenzoate to inhibit completely the activity of the succinic oxidase system. The bands did not appear, however, when succinate was used instead of $Na_2S_2O_4$, showing the absence of an active succinic dehydrogenase. When denatured globin was added to the tube containing succinate and the heart-muscle preparation treated with the mercurial, the

* Under the same conditions, the enzyme treated with one third as much arsenical (i.e. 0·5 μequiv./ml. of heart-muscle preparation) was reactivated by 25% by treatment with globin. The succinic dehydrogenase and the complete succinic oxidase system were reactivated to the same extent.

bands of the cytochromes immediately became visible. Thus the spectroscopic observations are in agreement with the manometric experiments.

DISCUSSION

Inactivation of succinic dehydrogenase by sulphydryl-combining reagents and reactivation by sulphydryl-containing compounds

Under the conditions of the experiments described above, the reaction between the enzyme and the arsenical or *o*-iodosobenzoate was slow. The slowness of the reaction must be considered when comparing the activity of different arsenicals. Barron, Miller, Bartlett, Meyer & Singer (1947) found that lewisite was much more effective than other organic arsenicals or inorganic arsenite in inhibiting the succinic oxidase system. If all arsenicals combine with the same groups on the enzyme to form a difficultly dissociable complex, one would expect all arsenicals to be equally effective, if they are allowed to react with the pure enzyme for a sufficient length of time. The differences found by Barron, Miller, Bartlett, Meyer & Singer may be due to differences in the rates of combination, not in the capacity for combination, or to differences in the relative rates of combination of the arsenical with the succinic dehydrogenase compared with the rate of combination with other proteins which are present in the enzyme preparation and which protect the enzyme from inactivation. These points require further investigation. It should be noted that the present finding, that the arsenical reacts slowly with the enzyme, is not in agreement with Barron & Singer (1945), who found that the inhibition was complete after 6 min. Many of the findings of the present communication do not agree with those of Barron and his co-workers. This may be due to the different method employed in studying the inhibition and perhaps also to the different enzyme preparation used (Barron & Singer (1945) used a preparation made by treating washed skeletal muscle in a Waring blender with phosphate buffer; it was not purified by acid precipitation). The concentration of the enzyme during treatment with the SH-combining reagents in the present studies was about 100 times that used by Barron & Singer (1945).

The finding that GSSG reacts with the enzyme very slowly, and that high concentrations and long incubation times are necessary to obtain an appreciable inhibition, is in agreement with the original observations of Hopkins *et al.* (1938). This is probably due to the difficulty of penetration of the large molecule of GSSG (mol. wt. 612) to the site of the inhibition. Keilin & Hartree (1940) found that disulphides such as tetraethyldithiocarbamyldisulphide and tetrathionate, whose molecular structure resembles that of pyrophosphate which is probably a competitive inhibitor of succinic dehydrogenase, are much more powerful inhibitors than GSSG possibly because they can penetrate more readily to the active centre of the enzyme which may be the site of the inhibition.

It is customary to express the effectiveness of an inhibitor of an enzyme in terms of the concentration of the reagent which produces a certain inhibition. This implies that the degree of inhibition is, in fact, determined by the concentration of the inhibitor when the enzyme activity is measured and not by the concentration of the enzyme. This is the case when the inhibitor enters into a loose easily reversible combination with the enzyme and the inhibition is reversed by dilution. The inhibitors, with which we are concerned in this paper, do not, however, form such an easily reversible compound, but take part in a chemical reaction which proceeds practically to completion, and the compound formed is not dissociated by subsequent lowering of the concentration of the inhibitor by dilution. The degree of inhibition depends then, not on the final concentration of the inhibitor in the enzyme solution when the activity is measured, but on the ratio of the concentration of the inhibitor to that of the enzyme provided that sufficient time is allowed for the reaction to proceed to completion. The actual concentrations of the reactants will, of course, affect the rate at which this point is reached, and become more important than the ratio of the concentrations when the reaction is very slow, for example, when GSSG is the inhibitor.

If the effect of *o*-iodosobenzoate is to oxidize SH groups in the enzyme molecule to S.S, it is not surprising that removal of the inhibitor should have no effect on the inactivated enzyme. It is surprising, however, that, after treatment with the arsenical, the enzyme cannot be reactivated by removing the untreated arsenical and washing with water. It is generally believed that the reaction of the enzyme with the arsenical is

$$\begin{matrix}-SH\\-SH\end{matrix} + O=As-R \rightleftharpoons \begin{matrix}-S\\-S\end{matrix}\!\!>As-R + H_2O. \quad (1)$$

Enzyme Arsenical Enzyme-arsenical compound

If this is the case, one would expect that removal of the arsenical would cause some dissociation of the inactive enzyme-arsenical compound, but this does not appear to occur. No reactivation was obtained by diluting a partially inactivated enzyme preparation 55-fold even after keeping it 3 hr. before measurement of the succinic oxidase activity. The equilibrium of the above reaction must lie far to the right. In this respect, succinic dehydrogenase resembles kerateine; Stocken & Thompson (1946*a*) found that 85% of the arsenic combined with the kerateine remained after precipitation at pH 4·6 and washing.

p-Chloromercuribenzoate differs from the other agents used in several important respects, viz. (*a*) it inactivates the enzyme more rapidly; (*b*) it does not react with other substances in the enzyme preparation before attacking the enzyme; (*c*) its action is readily reversed by GSH or denatured globin, but not by BAL; the action of the arsenical is, on the other hand, reversed much more effectively by the dithiol than by the monothiol or globin, and BAL is also rather more effective than GSH in reversing the effect of *o*-iodosobenzoate.

These differences are probably all connected with the fact that the mercurial combines with only one SH group thus,

$$—SH + R—Hg—Cl \rightarrow —S—Hg—R + HCl, \quad (2)$$

while the arsenical combines with two such groups (either on the same or on different enzyme molecules) as shown in reaction (1) and the oxidizing agent also requires two SH groups to form a disulphide

$$\begin{matrix} —SH \\ —SH \end{matrix} + R—I=O \rightarrow \begin{matrix} —S \\ | \\ —S \end{matrix} + R—I + H_2O. \quad (3)$$

It is readily understandable that the reaction with one SH group might be more rapid than that with two SH groups. The second point of difference between the mercurial and the other inhibitors probably follows from this difference in the rates of the reaction, if it is assumed that the enzyme preparation contains substances which, unlike the enzyme, are readily attacked by both types of inhibitor. The third point of difference can be explained by assuming that the mercurial readily combines with monothiols, but not with dithiols like BAL. Arsenicals, on the other hand, are known from the work of Stocken & Thompson (1946*b*), extended by Whittaker (1947), to combine much more easily with dithiols, with which they can form stable rings containing the arsenic and the sulphur. GSH and BAL do not differ so markedly in their capacity to reverse inhibition by iodosobenzoate, and the greater activity of BAL in this case may be due to the stronger reducing properties of this compound. Denatured globin behaves, in respect of its ability to reverse inhibition by the mercurial and the arsenical, like a monothiol. Mirsky & Anson (1936) have shown that denatured globin, like other denatured proteins, contains a number of reactive SH groups.

It is interesting to note that Barron & Kalnitsky (1947) have found that the inhibiting action on the succinic oxidase system of mercuric chloride, which would be expected to react more readily with dithiols (it gives an insoluble precipitate with BAL) than with monothiols, is reversed much more readily by dithiols than by monothiols.

From the point of view of the position of the final equilibria, the reactivation of the enzyme inhibited by arsenicals can be explained by competition between the BAL and the enzyme for the arsenical according to the equations.

$$\begin{matrix} —SH \\ —SH \end{matrix} + O=As—R \rightleftharpoons \begin{matrix} —S \\ —S \end{matrix}\!\!>\!As—R + H_2O, \quad (4)$$

$$\begin{matrix} CH_2—SH \\ | \\ CH—SH \\ | \\ CH_2—OH \end{matrix} + O=As—R \rightleftharpoons \begin{matrix} CH_2—S \\ | \\ CH—S \\ | \\ CH_2—OH \end{matrix}\!\!>\!As—R + H_2O. \quad (5)$$

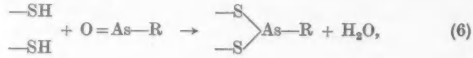
However, such an explanation introduces a kinetic difficulty. It has already been shown that the reaction between the enzyme and the arsenical is slow and that the position of the equilibrium in reaction (4) is far towards the right. It must follow that the reverse reaction (4) is still slower than the forward; consequently no matter how fast the reaction between BAL and the arsenical, the rate of dissociation of the enzyme-arsenical compound would be the reaction limiting the reactivation of the enzyme, which would therefore be very slow. However, contrary to this conclusion, it was found that the reactivation was much more rapid than the inactivation.

This suggests that the above equations do not correctly express the reactions involved. Two possible modifications are as follows:

(*a*) The process limiting the rate of inactivation is not the actual combination between the enzyme and the arsenical, but the rate of diffusion of the arsenical to the reactive groups of the enzyme. Thus the actual velocities of the forward and reverse reactions (4) may be much greater than indicated by the slowness of the inactivation. According to this view, the relatively rapid reactivation is due to the ease of penetration of BAL to the site of the inhibition, and to the fact that the arsenical-BAL compound diffuses more rapidly than the arsenical. This explanation is supported by the ability of BAL to penetrate rapidly into tissues and thereby combine with lewisite some time after contact of the tissue with the lewisite (Stocken & Thompson, 1946*b*), and by the finding of Peters & Stocken (1947) that the compound between BAL and mapharside (which is very similar to the arsenical used in the present investigation) is more toxic to rats than the arsenical itself, presumably owing to readier penetration of the compound to sites which are dangerous to the animals.

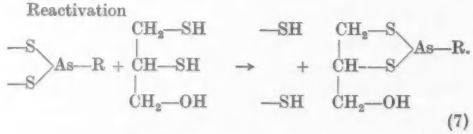
(*b*) The reactivation is not due to competition between the enzyme and the BAL for the arsenical formed by dissociation of the enzyme-arsenical compound, but essentially irreversible reactions are involved, both in inactivation and reactivation.

Inactivation



(6)

Reactivation



(7)

Is succinic dehydrogenase a monothiol or a dithiol?

Dithiols were introduced as antidotes in arsenical poisoning by Peters *et al.* (1945). Stocken & Thompson (1946*b*) found that the inhibition of the pyruvic oxidase system by arsenicals was completely reversed by dithiols, while monothiols were ineffective, and suggested that this was due to the fact that the arsenical combines with two essential SH groups, closely situated in the enzyme molecule, so as to form a relatively stable cyclic structure. Since the dithiol BAL is also much more effective than GSH in reversing the inhibition of succinic dehydrogenase by *p*-aminophenylarsenoxide, it might appear justifiable to conclude that the succinic dehydrogenase molecule also contains two closely situated SH groups.

Before considering this matter, it should be noted that there are important differences between the pyruvic and succinic enzymes with regard to inhibition by arsenicals and reversal by thiols, as has been pointed out by Thompson (1948). Thus, Stocken & Thompson (1946*b*) have shown that monothiols are completely ineffective in preventing or reversing the action of arsenicals on the pyruvic enzyme, whereas Barron, Miller, Bartlett, Meyer & Singer (1947) and the present investigation have shown that GSH is able to reverse partially the inhibition of the succinic enzyme, although it is much less effective than BAL. In addition, it has been shown by Peters, Sinclair & Thompson (1946) that in brain preparations containing both enzymes, the pyruvic is much more susceptible than the succinic enzyme to arsenicals.

It does not necessarily follow from the greater effectiveness of dithiols compared with monothiols in reversing the inhibition by arsenicals that the enzyme is, itself, a dithiol. The weakness of this type of argument is shown by the fact that if applied to the findings with the arsenical it would be concluded that the succinic enzyme was a dithiol, while if applied to the experiments with the mercurial, the conclusion drawn would be that the enzyme was a monothiol. As it is the same enzyme in both cases, the different results obtained must be a property of the inhibitor, not of the enzyme. Since Stocken & Thompson (1946*b*) have shown that dithiols form more stable compounds with the trivalent arsenicals than do monothiols, it would be expected that the dithiols would be more satisfactory reactivating agents, regardless of the nature of the combination between the arsenical and the enzyme.

Thus it is not permissible to conclude, from the experiments on the reactivation of the enzyme inhibited by arsenicals, that the enzyme contains two SH groups which react with the same arsenic atom. The other findings, in fact, without definitely settling the matter, suggest that it is more likely that the enzyme contains only one SH group necessary for activity, since it is difficult to reconcile otherwise the great ease of inhibition by the mercurial with the failure of BAL to reactivate after such inhibition, which shows that the mercurial does not readily react with compounds containing two closely situated SH groups. The finding that BAL in an amount equivalent to the amount of arsenical combined in the enzyme almost completely reactivated the enzyme, showing that the arsenical forms a very much firmer union with the simple dithiol than with the enzyme, is also in agreement with this view. If the enzyme contains only one essential SH group, it is probable that the arsenical and oxidizing agents combine with two SH groups from different molecules, only one of which may be the group on the enzyme necessary for its activity.

Components of the succinic oxidase system inhibited by SH-combining reagents

It has been shown that the inhibition of the complete succinic oxidase system by the arsenical can be accounted for by the inhibition of the succinic dehydrogenase. The simplest explanation of the action of arsenicals is that they combine with two SH groups, either both in the same succinic dehydrogenase molecule or on different molecules, and it would be expected that SH groups capable of reacting in this way would be oxidized by oxidizing agents to disulphides. However, the fact that the complete succinic oxidase system is affected to a greater extent than the succinic dehydrogenase shows that the latter is not the only point of attack by oxidizing agents on the system. The curve shown in Fig. 2 indicates that two reactions are proceeding, even at room temperature, and the further oxidation might proceed even more readily at 38°. The other part of the system which is attacked can also be reactivated by treatment with thiols, but with much greater difficulty than is the succinic dehydrogenase. The fact that the action of an oxidizing agent can be reversed by treatment with SH compounds is often taken as an indication that the oxidizing agent has attacked SH groups. However, this does not necessarily follow. Other groupings besides SH groups might be oxidized by oxidizing agents and reduced by subsequent treatment with reducing agents. It seems preferable to consider that SH

groups are attacked only when the enzyme is inhibited by a variety of agents, viz. alkylating agents —such as iodoacetate or chloroacetophenone— arsenicals and mercaptide-forming agents. In this case, the other portion of the system is not attacked by arsenicals. It has been shown that the cytochrome oxidase activity is not affected by oxidizing agents. It seems possible then, that the oxidizing agents affect, in addition to the succinic dehydrogenase, the component of the succinic oxidase system which links the dehydrogenase to cytochrome *c*, and, as is discussed elsewhere (Slater, 1949*c*), is destroyed by coupled oxidation with BAL or GSH. However, the possibility that the oxidizing agents, in addition to their effect on SH groups, have the non-specific type of effect on the system discussed by Keilin & Hartree (1949) and Slater (1949*d*) cannot be excluded.

It is interesting that treatment of the enzyme with GSH, in the absence of air but in the presence of GSSG, increased the inhibition of the succinic oxidase system while the succinic dehydrogenase was completely reactivated. It is possible that GSSG, in the high concentration used in this experiment, may be able to replace oxygen in promoting the destruction by BAL of the component of the system mentioned in the preceding paragraph. However, this point requires further clarification.

It is somewhat surprising that the enzyme, inhibited by oxidizing agents, is not reactivated by potassium cyanide (0·01 M), since Walker (1925) showed that cyanide reduces disulphides. Possibly, higher concentrations of cyanide would be effective.

The effect of *p*-chloromercuribenzoate is rather complicated. It is clear that it attacks the complete system more readily than the succinic dehydrogenase portion. This is in agreement with the work of Cook & Perisutti (1947) and of Cook, Kreke, McDevitt & Bartlett (1946) on the effect of phenylmercuric nitrate on the succinic oxidase system, although with this reagent these workers found a greater difference between the two inhibitions. *p*-Chloromercuribenzoate inhibited cytochrome oxidase, but this is insufficient to account for the greater effect on the complete succinic oxidase system than on the succinic dehydrogenase, since the cytochrome oxidase is not the limiting factor in the succinic oxidase system in the heart-muscle preparation. Cook *et al.* (1946) also found an inhibition of the cytochrome oxidase activity with phenylmercuric nitrate. They found, in addition, that other enzymes, viz. lactic dehydrogenase and catalase, which do not usually react with SH-combining reagents, were inhibited by this compound. This suggests that these mercurials have a non-specific effect on the entire succinic oxidase system as well as combining with SH groups in the dehydrogenase.

The function of the sulphydryl groups in succinic dehydrogenase

Since succinate or competitive inhibitors of succinic dehydrogenase (which are assumed to combine with the enzyme at the same point as succinate) protect the enzyme from the action of SH combining reagents, Hopkins & Morgan (1938) suggested that the SH group is at or near the point where succinate attaches itself to the enzyme. However, since succinate was unable to reduce succinic dehydrogenase treated with GSSG, these authors concluded that oxidation of SH to S.S did not play any part in the catalysis of the oxidation of succinate. Potter & Dubois (1943), on the other hand, have ascribed such a catalytic function to the SH group, which is presumed to mediate the transfer of electrons from succinate to cytochrome oxidase, by oscillating between the EnSH and EnS. forms. They suggest that the SH group lies between two 'carbonyl affinity groups', which form hydrogen bonds with the carbonyl oxygen atoms of succinic acid. By means of atomic models, Potter & Dubois were able to show that the combination of the type they envisage would result in the hydrogen atoms of succinate being directed towards the SH group, while the hydrogen atoms of malonate would be directed away, thus explaining the fact that malonate, although combining with the enzyme, is not oxidized. Although this theory of Potter & Dubois satisfactorily explains some of the properties of the enzyme, a further examination to see if it can explain the specificity of the enzyme and competitive inhibition by oxaloacetate and pyrophosphate seems to be required.

Unpublished experiments of the author have raised some doubt whether it is even permissible to conclude from the experiments of Hopkins & Morgan (1938) and Potter & Dubois (1943) that the SH group is near the point where the succinate is attached to the enzyme molecule. It was found that succinate and competitive inhibitors will protect the succinic oxidase system from destruction by BAL, although the point of attack on the succinic oxidase system is not even on the succinic dehydrogenase, but on a completely separate component. This suggests that the attachment of succinate or a competitive inhibitor to the succinic dehydrogenase has an effect extending beyond the succinic dehydrogenase molecule itself. It is known that the separate components of the succinic oxidase system must be closely associated with one another, so that each component is easily accessible to those with which it reacts.

SUMMARY

1. Inhibition of the succinic oxidase system by *p*-aminophenylarsenoxide, *o*-iodosobenzoate and oxidized glutathione is a slow reaction; *p*-chloromercuribenzoate reacts much more rapidly.

2. The heart-muscle preparation contained substances which protected the succinic oxidase system from small amounts of *p*-aminophenylarsenoxide and *o*-iodosobenzoate, but not of *p*-chloromercuribenzoate.

3. *p*-Aminophenylarsenoxide acts on the succinic oxidase system by inhibiting the succinic dehydrogenase. Oxidizing agents (*o*-iodosobenzoate, oxidized glutathione and cupric salts) have a more pronounced effect on the succinic oxidase system than on the succinic dehydrogenase, possibly by oxidizing the factor linking succinic dehydrogenase with cytochrome oxidase. *p*-Chloromercuribenzoate also affects the succinic oxidase system more than the succinic dehydrogenase; in addition, it has some effect on the cytochrome oxidase. It is likely that inhibition of the succinic oxidase system by *p*-chloromercuribenzoate is partly due to a non-specific effect on the entire succinic oxidase system.

4. After treatment with sulphydryl-combining reagents, the enzyme was not reactivated by dilution or by precipitation of the enzyme and washing away the excess inhibitor.

5. After inhibition by *p*-aminophenylarsenoxide, the enzyme was rapidly reactivated by small concentrations of BAL. Reduced glutathione was less effective and denatured globin only slightly effective as reactivating agents. The succinic dehydrogenase and the succinic oxidase system were reactivated to the same extent.

6. After inhibition by iodosobenzoate, the succinic dehydrogenase was almost completely reactivated by high concentrations of BAL. Reduced glutathione was slightly less effective. The succinic oxidase system was less readily reactivated than the succinic dehydrogenase.

7. BAL readily reactivated the succinic dehydrogenase after partial inhibition by oxidized glutathione, but increased the inhibition of the succinic oxidase system. Cyanide did not reactivate the enzyme inhibited by oxidized glutathione.

8. Succinic dehydrogenase inhibited by *p*-chloromercuribenzoate was largely reactivated by 0·01 M-cyanide. The succinic oxidase system, after treatment with the mercury compound, was readily reactivated by reduced glutathione and denatured globin, but not by BAL.

9. It is suggested that the action of these inhibitors on the succinic oxidase system be expressed in terms of the amount of inhibitor required to react with a definite amount of the enzyme preparation, not by the concentration of inhibitor required to produce a certain effect.

10. The differences between *p*-chloromercuribenzoate and the other inhibitors can partly be explained by the fact that this compound reacts with one sulphydryl group, while the other compounds require two sulphydryl groups.

11. The available evidence is not sufficient to decide definitely whether succinic dehydrogenase contains one or two sulphydryl groups which are necessary for its activity, although one is the more likely.

I would like to thank Prof. D. Keilin, F.R.S. for his interest and advice, Prof. R. H. S. Thompson for providing the BAL, Dr S. V. Perry for supplying the *p*-chloromercuribenzoate and the British Council for a scholarship.

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The Relationship between the Constitution and the Effect of Chemical Compounds on Plant Growth

1. 2-PHENOXYETHYLAMINE DERIVATIVES

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The discovery of the selective herbicidal effect of the substituted phenoxyacetic acids (Slade, Templeman & Sexton, 1945; Templeman & Sexton, 1946*a*) has prompted a further investigation of related compounds, with particular regard to the origin and function of 3-indolylacetic acid in green plants. The auxins of green plants exist only to a limited extent in the free state, the greater portion being present either as complexes, possibly of a proteinaceous nature, or as physiologically inactive precursors. It is generally believed that 3-indolylacetic acid in green plants, as in bacteria, originates from tryptophan, and direct evidence of this has been provided by the finding of Wildman, Ferri & Bonner (1947) that infiltration of tryptophan to spinach leaves greatly increased the amount of ether-extractable 'auxin' in a few hours. Oxygen was required in this process, and there was evidence that a carbonyl compound was an intermediate metabolite. Infiltration of 3-indolylpyruvic acid produced the same effect, but this was not so with tryptamine. It appears to have been generally assumed, without much direct evidence, that the pathway from tryptophan to 3-indolylacetic acid is the same in plants as in bacteria, proceeding through 3-indolylpyruvic acid by the route indicated in Fig. 1. It is theoretically possible, however, that the first step might be decarboxylation to tryptamine, followed by enzymic conversion to 3-indolylacetic acid, perhaps via the aldehyde. It is also possible that more than one route may operate or that different plant species use different routes. Thus, although the experiments of Wildman *et al.* (1947) using spinach may be taken as evidence against the tryptamine route, it has been shown by Skoog (1937) that tryptamine can be converted by cut surfaces of *Avena* to a substance having auxin activity. Decarboxylation of amino-acids may be inferred to

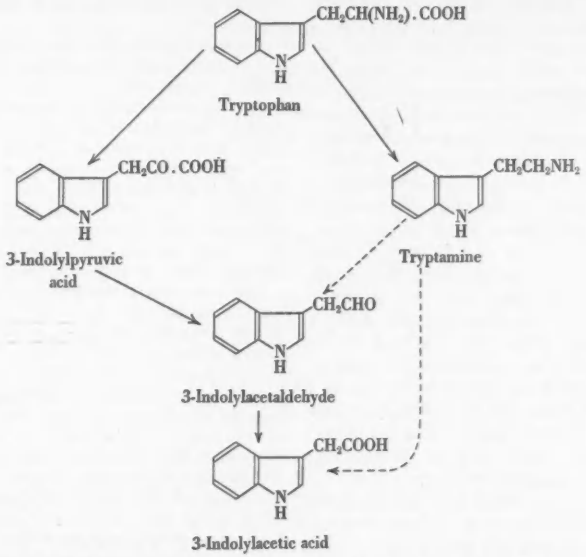


Fig. 1. Alternative pathways from tryptophan to β-indolylacetic acid.

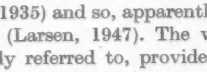
occur in plant tissue as several appropriately constituted amines have been recognized in the free state. Indeed, not only is the molecule of tryptamine discernible in such alkaloids as harmine, physostigmine and dipterine, but it has been found in the free state in certain *Acacia* species (White, 1944). From this it may be concluded that the tryptamine route to 3-indolylacetic acid is worth serious consideration, at least in certain plant species. Finally, it should be added that evidence for the occurrence in plants of 3-indolylacetaldehyde has been produced by Larsen (1944, 1947).

The work here described was based upon the following hypothesis. Plant growth is regulated, perhaps in part, through the maintenance of a critical level or concentration of 3-indolylacetic acid, and this level is maintained in a dynamic system by the balance of anabolic and catabolic processes. Degradation of 3-indolylacetic acid is known to occur in plants, for a degradative enzyme has been isolated from the pea epicotyl (Tang & Bonner, 1947). Any disturbance of this balance will result in hormonal dysfunction which may be revealed by such symptoms as epinasty, organ modification or cessation of growth. It follows from this hypothesis that the normal precursors of 3-indolylacetic acid may, by conversion to the latter compound, produce the same symptoms as 3-indolylacetic acid itself.

Tryptophan itself, although inactive by the *Avena* test for auxin (Link & Eggers, 1943) is, as stated above, convertible to auxin, and it inhibits root growth in cress (Audus & Quastel, 1947). 3-Indolylpyruvic acid is active in the *Avena* test (Kögl & Köstermans, 1935) and so, apparently, is 3-indolylacetaldehyde (Larsen, 1947). The work of Skoog (1937), already referred to, provides one piece of evidence for the conversion of tryptamine to 'auxin'. Our colleague, Dr A. Rhodes of Jealotts Hill Research Station, has found that tryptamine has a marked effect in reducing the root length of germinating cress and a much less marked effect on wheat. We have confirmed this observation (cf. also Audus & Quastel, 1947). Since certain substituted phenoxyacetic acids produce growth effects similar to those of 3-indolylacetic acid, we decided to study the problem further by moving from the indole series to the substituted 2-phenoxyethyl series, where syntheses are much more easy, and by employing the technique of seed germination.

METHODS

Preparation of compounds. The compounds selected for study were the substituted 2-phenoxyethylamines of the general formula



in which the benzene ring contained appropriate substituents and in which R was hydrogen or a hydrocarbon residue. The methods employed were conventional and are given here in outline only, details of the properties of the new compounds being given in Table 1, together with such analyses as were deemed desirable to check the purity of individual compounds or of a typical member of a series prepared by a general method.

The amines were made by interaction of the 2-phenoxyethylbromide with excess of NH_3 or the appropriate primary or secondary amine. When the latter was volatile, solutions in methanol under pressure were employed. In the case of 2-2′:4′-dichlorophenoxyethylamine a more convenient laboratory method was provided by the decomposition, by means of boiling ethanolic HCl, of the quaternary salt of the phenoxyethylbromide with hexamethylenetetramine.

Quaternary salts were prepared either by quaternization of the tertiary amines with alkyl halide or by reaction of the 2-phenoxyethylbromide with the appropriate tertiary amine. Quaternary salts of 2-phenoxyethylbromides with hexamethylenetetramine were obtained by reaction of the components in hot $CHCl_3$ solution, from which the crystalline salts separated.

Biological methods. The general requirement for this work was a rapid routine test method which would distinguish readily between active and inactive compounds without necessarily providing accurate quantitative data. Two plant species, rape and oats, were chosen as test objects, since the earlier work of Templeman & Sexton (1946*a*, *b*) had shown that several monocotyledonous species were susceptible to *iso*propyl phenylcarbamate, while some dicotyledonous species were generally the more susceptible to auxin-like herbicides, such as the substituted phenoxyacetic acids. The chosen seeds were oats (*Avena sativa*, var. White Winter) and rape (*Brassica campestris*, var. English Broad Leaved).

The seeds were allowed to germinate on agar slopes in 6 in. × ½ in. test tubes, each tube containing three rape seeds or two oat seeds. The number of tubes used was 12 for rape or oats at 100 p.p.m. and for rape at 50 p.p.m., 18 for rape at 10 p.p.m. or less and for oats at 50 p.p.m. or less. The tubes were mounted vertically in the dark at room temperature, and the root lengths measured when the rape roots were 5–6 cm. long (5–7 days) and the oats 6–7 cm. long (8–10 days). Seeds which failed to germinate were rejected, and the average root length ('average control length') of the remaining seeds was measured. With oats, the longest root produced by each seed was measured, since trial experiments had shown this to be reasonably proportionate to the combined lengths of all the roots. In the experimental tubes, the substance under test was incorporated with the agar at a concentration of 100 p.p.m. and greater dilutions were examined subsequently if necessary. The seeds were examined at the same time as the controls, and the effect of the chemical was assessed in the following way. Those substances which caused more than half the roots to be less than 50% of the average control length were characterized as 'very active' and are given two plus signs in the tables. If a substance failed to reduce half the roots to less than 50% of the average control length it was then reassessed on a basis of 80% of the average root length. If more than half the roots were reduced to 80% of the average control length, the substance was marked with one plus sign and characterized as 'active'. If it failed to reduce half the roots to 80% of the average control length, it was characterized as inactive (–).

Table 1. *New compounds synthesized*

Formula	Physical characteristics	Analyses	Found (%)	Required (%)
$C_6H_5.O.CH_2.CH_2.NH_2$	Hydrochloride sinters at 180°, m.p. 210°	(Hydrochloride)	N, 8·0	8·05
p-$Cl.C_6H_4.O.CH_2.CH_2.NH_2$	Colourless oil, b.p. 152–156°/15 mm. Hydrochloride, m.p. 228°	(Hydrochloride)	N, 6·8	6·7
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NH_2$	Pale yellow oil, b.p. 174–176°/15 mm. Hydrochloride, m.p. 188–192°	(Base)	C, 46·8 H, 4·55	46·6 4·4
		(Hydrochloride)	Cl, 44·2 N, 5·8	43·8 5·8
1:3:4-$C_6H_3Me_2.O.CH_2.CH_2.NH_2$	Colourless oil, b.p. 140°/15 mm. Hydrochloride, m.p. 190°	(Hydrochloride)	N, 7·2	7·0
1:5:2-$C_6H_3MeCl.O.CH_2.CH_2.NH_2$	Colourless oil, b.p. 154–156°/15 mm. Hydrochloride, m.p. 162–164°	(Hydrochloride)	N, 6·3	6·3
2:4:5:1-$C_6H_2Cl_3.O.CH_2.CH_2.NH_2$	Colourless oil, b.p. 170°/15 mm. Hydrochloride, m.p. 237–239°	(Hydrochloride)	C, 35·2 H, 3·35	34·7 3·25
2-$C_{10}H_7.O.CH_2.CH_2.NH_2$	Hydrochloride chars at 260°	(Hydrochloride)	N, 6·5	6·3
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NHMe$	Colourless oil, b.p. 160°/15 mm.	(Hydrochloride)	N, 5·35	5·45
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2$	Colourless oil, b.p. 160–164°/12 mm. Hydrochloride hygroscopic	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NEt_2$	Liquid, b.p. 100°/0·15 mm. Hydrochloride, m.p. 127–128°	(Hydrochloride)	N, 4·5	4·7
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NHC_6H_5$	M.p. 42–44°. Hydrochloride decomposed by water, m.p. 137–140°	(Base)	C, 59·8 H, 4·8 N, 5·4	59·7 4·6 5·0
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMeC_6H_5$	Pale yellow viscous oil, b.p. 250–260°/15 mm. Insoluble in dilute HCl	—	Cl, 24·05	24·2
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N(CH_2.CH_2)_2CH_2$	Pale yellow oil, b.p. 194–196°/15 mm. Hydrochloride, m.p. 158–160°	(Hydrochloride)	N, 4·8	4·5
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N(CH_2.CH_2)_2O$	Base: thick oil, b.p. 210–212°/15 mm. Hydrochloride, m.p. 86°	(Hydrochloride)	N, 4·45	4·5
$(2:4:1\text{-}C_6H_3Cl_2.O.CH_2.CH_2.)_2NH$	Hydrochloride, m.p. 183–185°	(Hydrochloride)	N, 3·25	3·2
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_3^+Br^-$	White crystals, m.p. 88–89°	—	C, 39·25	40·0
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2Et^+I^-$	M.p. 77–78°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_2.CH_3^+Br^-$	M.p. 146–148°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_3.CH_3^+Br^-$	M.p. 194°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_5.CH_3^+Br^-$	M.p. 160–162°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.NMe_2.(CH_2)_6.CH_3^+Br^-$	M.p. 160–162°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_{11}.CH_3^+Br^-$	M.p. 120–124°	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NEt_3^+I^-$	—	—	—	—
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe(CH_2.CH_2)_2CH_2^+I^-$	M.p. 158–160°	—	C, 40·7 H, 4·45	40·4 4·8
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (from hexamethylenetetramine)	M.p. 164°	—	N, 13·8	13·7
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NC_5H_5^+Br^-$ (from pyridine)	M.p. indefinite (hygroscopic)	—	—	—
1:3:4-$C_6H_2Me_2.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (from hexamethylenetetramine)	M.p. 176–179°	—	—	—

Table 2. *Herbicidal activity of standard substances*

(The activities in Tables 2–5 are indicated by + and − signs as explained in the text, p. 144.)

. means not examined

Compound	Formula	Concentration (p.p.m.)	Activity	
			Rape	Oats
4-Chloro-2-methylphenoxyacetic acid	2:4:1-$C_6H_3MeCl.O.CH_2.COOH$	20	.	++
		10	.	+
		0·1	++	.
2:4-Dichlorophenoxyacetic acid	2:4:1-$C_6H_3Cl_2.O.CH_2.COOH$	0·1	++	.
2-Naphthoxyacetic acid	2-$C_{10}H_7.O.CH_2.COOH$	10	++	.
		1	++	.
*iso*Propyl phenylcarbamate	$C_6H_5.NH.COO.CHMe_2$	0·1	−	++
Tryptamine	$C_8H_6N.CH_2.CH_2.NH_2$	1·0	++	.

Basic substances were examined as their hydrochlorides, acidic substances as their Na salts, while the quaternary ammonium compounds were all neutral salts, freely soluble in water at the required concentrations. Using this method of assessment, results obtained with known herbicidal substances are given in Table 2, thereby establishing a reference standard.

RESULTS

Systematic Testing. The results of the examination of the various substances are given in Tables 3–5, which are arranged so as to emphasize relationships between structure and activity. All concentrations are expressed in parts/million (p.p.m.).

Table 3. *Effect of substitution in the benzene ring upon the herbicidal activity of* 2*-phenoxyethylamine*

Compound ($R = CH_2.CH_2.NH_2$)	Concentration (p.p.m.)	Activity	
		Rape	Oats
$C_6H_5.OR$	100	−	−
1:4-$C_6H_4(OR)_2$	10	++	−
	2	++	−
	0·1	−	−
2:4:1-$C_6H_2Cl_2.OR$	10	++	−
	2	++	−
	0·1	−	.
1:5:2-$C_6H_3MeCl.OR$	5	++	−
	1	++	−
	0·1	−	.
1:3:4-$C_6H_3Me_2.OR$	50	++	−
	10	+	−
2:4:5:1-$C_6H_3Cl_3.OR$	50	++	−
	10	−	.
2-$C_{10}H_7.OR$	10	++	−
	2	−	.
1-$C_{10}H_7.OR$	50	+	+
4:1-$C_6H_4NO_2.OR$	50	−	−

Preliminary investigations regarding mode of action. If the working hypothesis is correct, the activity of chlorinated 2-phenoxyethylamine against rape is to be attributed to its conversion to the corresponding chlorinated phenoxyacetic acid. This might conceivably be brought about through an amine oxidase similar to that found abundantly in animal sources and described by Blaschko, Richter & Schlossmann (1937), and which is inhibited competitively by *iso*propylamine derivatives of the type of ephedrine. Tryptamine is known to be a substrate for this enzyme. Accordingly, attempts were made to determine whether the activity of 2:4-dichlorophenoxyethylamine could be antagonized by ephedrine. Ephedrine itself was inactive on both species at 100 p.p.m. and at this strength it failed to antagonize the effect of 5 p.p.m. of 2:4-dichlorophenoxyethylamine.

Veldstra & Havinga (1943) have advocated the hypothesis that substances with an auxin-like activity must be constituted with a suitable nucleus (e.g. indole, naphthalene or substituted benzene) from which an acidic group projects at an angle to the plane of the nucleus. According to this hypothesis, growth regulation by the auxins is a function of the negative pole of the acidic group, which affects ionic transport. It might perhaps be expected then that a similarly constituted molecule, but with a positive instead of a negative pole, would act in a manner antagonistic to that of the naturally functioning auxin or auxin-like substance. Tests with mixtures of 2:4-dichlorophenoxyacetic acid and 2-2′:4′-dichlorophenoxyethylamine on rape, however, showed no evidence of antagonism.

In order to obtain further evidence for the hypothesis that the phenoxyethylamines functioned through conversion to the phenoxyacetic acids, certain substances were submitted in a qualitative way to the pea test for auxin activity. This test was conducted as described by Went & Thimann (1937), but since quantitative results were not required, visual inspection was substituted for measurement of angles of curvature. Solutions were examined at a range of concentrations, and the results are given in Table 6.

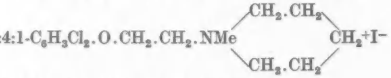
Table 4. *Effect of N-substitution (formation of secondary and tertiary amines) on the herbicidal activity of substituted 2-phenoxyacetic acids*

Compound	Concentration (p.p.m.)	Activity	
		Rape	Oats
2:4:1-$C_6H_2Cl_2.O.CH_2.CH_2.NH_2$	2	++	−
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NHMe$	50	++	−
	10	−	−
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2$	100	++	++
	50	++	+
	10	−	−
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NEt_2$	100	++	++
	50	−	+
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NH.C_6H_5$	100	++	+
	50	++	.
	10	+	.
	5	−	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe.C_6H_5$	100	++	−
	50	+	.
	10	+	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N$(CH$_2$.CH$_2$)$_2$CH$_2$	100	++	++
	50	+	++
	20	−	.
	10	−	+
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N$(CH$_2$.CH$_2$)$_2$O	50	++	++
	10	−	+
(2:4:1-$C_6H_2Cl_2.O.CH_2.CH_2.$)$_2$NH	50	++	++
	10	−	−
$C_6H_5.O.CH_2.CH_2.NH_2$	100	−	−
$C_6H_5.O.CH_2.CH_2.NH.C_6H_5$	100	+	+
$C_6H_5.O.CH_2.CH_2.NMe.C_6H_5$	100	−	+
2-$C_{10}H_7.O.CH_2.CH_2.NH_2$	10	++	−
	2	−	−
2-$C_{10}H_7.O.CH_2.CH_2.NH.C_6H_5$	100	−	+

DISCUSSION

Considering first the primary amines of Table 3, it is clear that those substituents in the benzene ring which favour activity against dicotyledonous species in the phenoxyacetic acid series (Templeman & Sexton, 1946*a*) also favour similar activity in the phenoxyethylamine series. The 4-chloro, 2:4-dichloro and 4-chloro-2-methyl derivatives were the most active of the compounds examined, the 2-naphthoxy compound was intermediate in activity, while the unsubstituted compound and the 4-nitro compound were inactive at the concentrations tested. In general, the amines did not appear to be quite as active as the corresponding acids. This finding constitutes evidence in favour of the hypothesis that these substances function in plant growth regulation through conversion to the phenoxyacetic acids. It has been demonstrated experimentally that they do not function as antagonists for the phenoxyacetic acids. Direct biochemical evidence for the hypothesis, however, is lacking. Blaschko *et al.* (1937) failed to find amine oxidase in certain green plants (seeds, tubers and leaves), but it does not follow from this that the enzyme is absent from germinating rape seed. It is possible, however, that a different enzyme system may be responsible for the degradation of tryptamine or the phenoxyethylamines in green plants, and this would account for our failure to antagonize the effect of 2:4-dichlorophenoxyethylamine by the amine oxidase inhibitor, ephedrine. Furthermore, the antagonism experiment would involve the question of the cell permeability of ephedrine, about which nothing is known. The failure to antagonize the effects of 2:4-dichlorophenoxyethylamine in this manner does not therefore invalidate the hypothesis concerning its mode of action. Two further facts in support are to be noted. First, the activity of 2:4-dichlorophenoxyethylamine in the pea test is of a similar nature to that of 2:4-dichlorophenoxyacetic acid though it differs quantitatively. Secondly, a preliminary observation in the

Table 5. *Herbicidal activity of quaternary derivatives of substituted 2-phenoxyethylamines*

Compound	Concentration (p.p.m.)	Activity: Rape	Activity: Oats
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_3^+Br^-$	50	−	+
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2Et^+I^-$	50	+	++
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_2.CH_3^+Br^-$	50	++	+
	10	+	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_3.CH_3^+Br^-$	50	++	+
	10	++	.
	5	+	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_6.CH_3^+Br^-$	50	++	−
	10	++	.
	5	+	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NMe_2.(CH_2)_{11}.CH_3^+Br^-$	50	−	−
	50	+	++
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NEt_3^+I^-$	50	++	++
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (*a*)	50	++	+
	10	++	−
	5	++	.
2:4:1-$C_6H_3Cl_2.O.CH_2.CH_2.NC_5H_5^+Br^-$ (*b*)	20	+	++
	10	−	−
2:4:1-$C_6H_3Me_2.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (*a*)	50	++	+
	20	++	−
	10	−	.
p-Cl.$C_6H_4.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (*a*)	50	.	++
	10	++	.
2-$C_{10}H_7.O.CH_2.CH_2.N_4C_6H_{12}^+Br^-$ (*a*)	100	.	+
	50	+	.

(*a*) From hexamethylene tetramine.
(*b*) From pyridine.

field indicated a similar physiological response of growing buttercups to both the amine and the acid. Direct experiments on the conversion of the amine to the acid in plant tissue or by plant extracts have not yet been made.

Table 6. *Results of the pea test for auxin activity*

Compound	Concentration (p.p.m.)	Effect observed
2:4-Dichlorophenoxyacetic acid	1	Equal to 3-indolylacetic acid at 5 p.p.m.
2:4-Dichlorophenoxyethylamine	10	Equal to 3-indolylacetic acid at 1 p.p.m.
Quaternary compound of hexamethylenetetramine with 2:4-dichlorophenoxyethyl bromide	100	Equal to 3-indolylacetic acid at 5 p.p.m.

Turning now to the first three compounds in Table 4 it is seen that substitution of the amino hydrogen atoms by methyl groups decreases the activity towards rape and increases the susceptibility of oats. The fully methylated compound is apparently much more active against oats than is 2:4-dichlorophenoxyacetic acid. This can be interpreted in more than one way. The relative insusceptibility of oats to the phenoxyacetic acid or the primary phenoxyethylamines might be due to the failure of these compounds to penetrate in adequate amounts to the appropriate site of action; methylation might assist such penetration, thus causing the phenoxyacetic acid to be liberated at a place which would otherwise be inaccessible to this molecule. The oat plant can, of course, respond to the phenoxyacetic acids under appropriate circumstances, for they are active in the standard *Avena* coleoptile test for auxins. It is possible, though perhaps not very likely, that methylation has the reverse effect on permeability in rape, so that the activity against germinating rape seed is reduced. This question cannot be answered until much more is known of the permeability of different plant species and tissues to organic compounds. Another possibility is that the secondary and tertiary amines function by an entirely different mechanism in which the greater susceptibility of oats is inherent. On the whole, the results given in Table 4 show that with the secondary and tertiary amines generally, there is much less

difference in the susceptibility of the two species than with the primary amines. This caused us to take the further step of examining quaternary compounds.

Amongst the quaternary compounds of Table 5, the 2:4-dichloro series will be considered first. It can be seen that as the molecular weight in the series of *n*-alkyl compounds rises, the activity against rape increases to C_6 and C_7, but the C_{12} compound is inactive. This is the familiar peak effect in an homologous series which is found in many relationships between chemical constitution and biological activity. Against oats there appears to be a maximum at C_2, though the result with the C_6 compound is anomalous. In view of the possibility that these quaternary compounds may be functioning by a mechanism bearing no particular relation to the chlorinated phenoxy nucleus, a general investigation of the activity of quaternary salts is now in progress and will be reported separately.

The quaternary compounds of hexamethylenetetramine call for special comment. Such compounds are known to decompose in boiling neutral aqueous solution with generation of aldehydes according to an oxidation-reduction reaction which has recently been discussed by Angyal & Rassack (1948), and we have evidence of decomposition at room temperature. Attempts to convert the hexamethylenetetramine derivative of 2:4-dichlorophenoxyethyl bromide into the aldehyde met with mixed success. While boiling with water definitely gave rise to some aldehydic material, as judged by the formation of a bisulphite compound, the aldehyde itself could not be isolated and appears to be unstable; compare the instability of phenoxyacetaldehyde (Pomeranz, 1894).

Under acidic conditions these hexamethylenetetramine derivatives are decomposed to the primary amines. This reaction has been found to proceed well in the case of the 2:4-dichlorophenoxy derivatives. Thus it may be argued that the hexamethylenetetramine compounds are potential precursors of the phenoxyacetic acids in living systems. In accordance with this conception, both the 2-2′:4′-dichlorophenoxyethyl and 2-2′:4′-dimethylphenoxyethyl compounds of hexamethylenetetramine are active against rape at high dilution, and indeed the former has been shown to produce the characteristic response of the substituted phenoxyacetic acids with growing buttercups. It is possible therefore that these compounds owe their activity against oats to their quaternary structure, but that their high activity against rape is due to their conversion to the acids within the living organism.

(*Note added* 8 *April* 1949.) Since this paper was submitted for publication, confirmation of our hypothesis that indolylacetic acid can arise in plants from tryptophan either through indolylpyruvic acid or through tryptamine has been presented by Gordon & Nieva (1949). These authors have demonstrated the operation of the two alternative pathways in leaves of pineapple.

SUMMARY

1. The activity of substituted β-phenoxyethylamines against germinating oat and rape seed has been studied in the laboratory.

2. In the case of the primary amines, selective activity against rape is associated with the same benzene substituents as cause activity in the phenoxyacetic acid series.

3. Substitution in the amino group tends to alter the relative susceptibility of the two species, and there is evidence that the quaternary salt derivatives operate by a different mechanism.

4. Quaternary derivatives of appropriately substituted 2-phenoxyethyl bromides with hexamethylenetetramine are particularly active against rape.

5. The results are discussed in relation to a hypothesis that phenoxyethylamines are converted by the living plant to the phenoxyacetic acids.

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The Reduction of Gastric Acidity by Back-Diffusion of Hydrogen Ions Through the Mucosa*

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It is generally believed that the hydrochloric acid solution secreted by the oxyntic cells is isotonic with the blood, and that the reduction of the acidity observed in the gastric juice is due to subsequent dilution and neutralization by other secretions (see Babkin, 1944). Teorell (1933), using anaesthetized or decerebrate cats whose stomachs were tied at both ends, observed a rapid decrease in the acidity of hydrochloric acid solutions injected into the stomach, without a corresponding reduction of the volume of the injected fluid. From these results, and from model experiments with artificial membranes, he inferred that the reduction of the acidity of the gastric juice was due mainly to the diffusion of H^+ ions through the gastric mucosa into the blood. However, diffusion of H^+ ions through living gastric mucosa has not so far been demonstrated, and this paper describes an attempt to investigate the diffusion of H^+ ions through isolated amphibian gastric mucosa. The results show that H^+ ions can diffuse through surviving mucosa and that the rates of diffusion are linearly related to the H^+ ion concentration of the solution in contact with the secretory side of the tissue (see Teorell, 1933, 1939).

METHODS

Saline media. The salines, isotonic with frog blood, described by Davies & Terner (1949) were used. These were (*a*) a bicarbonate saline, in equilibrium with 5% CO_2+95% O_2, or with 5% CO_2+95% N_2, and (*b*) a phosphate saline, in equilibrium with 100% O_2. All salines contained 0·2% glucose.

Apparatus. The apparatus described by Davies & Terner (1949) was used in experiments with 'open sheets' of mucosa. It consists of a constant pressure manometer, a modified Warburg cup *B* and a special hollow stopper *S* to which a graduated capillary side tube *G*, is attached. A diagram of the apparatus is shown in Fig. 1 (Davies & Terner, 1949). Experiments with 'tied' mucosa were carried out in standard conical Warburg cups and manometers.

Analysis of solutions. All titrations and measurements of pH were done with the semi-micro electrometric titration unit described by Davies & Longmuir (1948).

* A part of this work has been communicated to the Biochemical Society (Terner, 1949).

Material. Toads (*Bufo bufo bufo* L.) and frogs (*Rana temporaria temporaria* L.) were captured locally and kept in glass tanks. An excess of worms was provided for food. The animal was killed and the stomach removed and washed with saline. The tube of mucosa was isolated by removing the muscle layer and prepared in two ways, to give either open sheets of mucosa or tied mucosa.

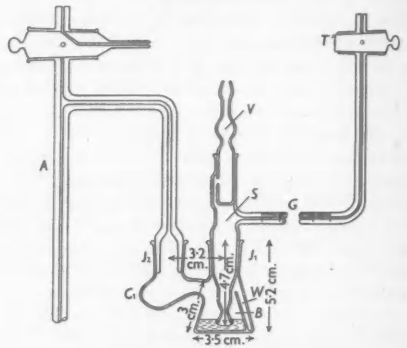

Fig. 1. Vessel *B*, hollow stopper *S*, with graduated side tube *G*, and part of manometer *A*. Capacity of *B*, 25 ml.; capacity of side arms C_1 and C_2 (C_2 is not shown), 1 ml. Outer diameter of *G*, 0·6 cm.; bore 0·1 cm., length 15 cm., graduation 1 division = 1 μl. The mucosa is tied to the base of *S* (diameter 0·9 cm.).

Open sheets of mucosa

The tube was opened by cutting along the lesser curvature (Davies, 1948) and the open sheet was tied with silk thread to the open base of a stopper *S* (Fig. 1) with the secretory side upwards. The secretory surface of the mucosa was covered with a solution of HCl which was pipetted into stopper *S*. The solutions contained 0·1, 0·05, 0·02 and 0·01 N-HCl and sufficient NaCl to make them isotonic with frog blood (0·12 M for univalent-univalent electrolytes). Stopper *S* and part of the graduated capillary side tube *G* were then filled with liquid paraffin, and the stopper was closed by inserting the tap stopper *V* (Fig. 1). The unit was placed in the main compartment of the vessel *B*, which contained 5 ml. of nutrient saline. The vessel was attached to the manometer and, after gassing, was placed in a water bath and shaken at 25·0°. An identical unit in which the mucosa was replaced by a rubber or plastic membrane was used as a thermo-

barometer. The manometers were of the constant pressure type, and the pressure inside the system could be adjusted so that a known resultant pressure was applied to the mucosa throughout the experiment. The pressure was measured in mm. manometric fluid (10,000 mm. manometric fluid = 1 atm.). (The manometric fluid in use in this laboratory, a modified Brodie solution, is prepared in the following way: 17·5 g. Na_2SO_4 (anhydrous), 5·0 g. sodium glycocholate, and 0·5 g. methyl violet, are dissolved in 500 ml. distilled water; 2–3 drops of capryl alcohol are added to prevent foaming. The solution is filtered before use.)

When pressure was applied in the direction of normal secretion (from the nutrient to the secretory side of the mucosa) it was called a positive pressure (e.g. +20 mm. manometric fluid); conversely, pressures opposing the direction of normal secretion were indicated by a negative sign (see Davies & Terner, 1949).

Readings were taken at 15 min. intervals and the changes in the volume of the gas in the manometer and the volume of fluid in stopper *S* recorded. The following measurements were made: (1) the rates of respiration (Q_{O_2}) and of fluid transport across the mucosa (q_{H_2O}) were measured in phosphate saline, gassed with 100% O_2, CO_2 being absorbed by NaOH in a glass pocket fused to the wall of vessel *B* (Fig. 1); (2) the rates of acid secretion (Q_{HCl}), or of diffusion of H^+ ions ($q^{H^+}_{CO_2}$) and the q_{H_2O}, were measured in bicarbonate saline gassed with 5% CO_2 + 95% O_2; (3) the sum of $q^{H^+}_{CO_2}$ and the $Q^{N_2}_{CO_2}$, and the q_{H_2O}, were measured in bicarbonate saline gassed with 5% CO_2 + 95% N_2. One mucosa was needed for each set of measurements.

Calculation of results. Since the secretion of liquid into stopper *S* during any period resulted in a loss of liquid from the cup *B*, allowance had to be made for this reduction of volume when calculating the gas exchanges. Thus, when the change of volume in the manometer was −10 μl., while the increase in the volume of liquid in the graduated capillary tube *G* was +2 μl., the uptake of gas was actually −10 + 2 = −8 μl. This was multiplied by a constant which depended on the gas used to give the volume of gas at N.T.P. (For theory of the apparatus see Davies & Terner, 1949.)

Atropine (final concentration 10^{-2} M) was added to the saline to reduce the contractions of the muscularis mucosae (Davies & Terner, 1949). Nevertheless, contractions occurred in 30% of the experiments; in these no reliable measurements of the rates of fluid secretion could be made. The measurement of the rates of gaseous exchanges was, however, not affected, since a contraction of the mucosa, although it produced an apparent uptake of gas by reducing the gas space of the manometer at constant pressure, also caused an apparent increase of the volume of fluid. Since allowance was made for these changes in calculating the gas exchanges the two effects cancelled out. Histamine (final concentration 5×10^{-5} M) and thiocyanate (final concentration 0·01 M) were added from the side arms.

After the experiment the mucosa was cut along the silk ligature, removed in two portions (circle and rim), washed in distilled water and dried overnight at 110°. The dry weight of the circular portion was used in the calculation of the $q^{H^+}_{CO_2}$, Q_{HCl} and q_{H_2O}. The Q_{O_2} was calculated by using the sum of the dry weight of the two portions. The $q^{H^+}_{CO_2}$, Q_{HCl} and Q_{O_2} are defined as μl. gas at N.T.P./mg. dry wt./hr., the q_{H_2O} as μl. transported fluid/mg. dry wt./hr. (In the case of $q^{H^+}_{CO_2}$, 1 g. ion H^+ = 22·4 l.)

In bicarbonate saline, the CO_2 uptake by gastric mucosa in excess of its basal gaseous exchanges is equivalent to the amount of acid produced (Davies, 1948). In this paper, $-Q_{CO_2}$ denotes the rate of uptake of CO_2 in excess of the basal gaseous exchange of the tissue. Similarly, $+Q_{CO_2}$ does not denote the rate of evolution of respiratory CO_2, but the rate of extra CO_2 evolution, due to liberation of gas from the bicarbonate saline by H^+ ions diffusing into it from the HCl solution across the mucosa. Since the basal gaseous exchanges result in only a small uptake of gas (about 5 μl./hr., i.e. less than the experimental error), this was neglected. Therefore $-Q_{CO_2} = Q_{HCl}$, and $+Q_{CO_2} = q^{H^+}_{CO_2}$.

Tied mucosa

The tube of gastric mucosa was tied with silk thread at both ends and incubated in Warburg cups containing 4 ml. of bicarbonate saline gassed with 5% CO_2 + 95% O_2 at 25·0° (Davies, 1948). The resultant of O_2 uptake and CO_2 output was measured with Warburg manometers. Secretion of HCl was induced by addition of histamine to a final concentration of 5×10^{-5} M. Readings were continued for periods up to 10 hr. to see whether the acid secreted by the mucosa itself would diffuse out of the bag in which it had accumulated during the secretory phase. At the end of the incubation period the distended bag was blotted and weighed. It was cut open, the contents collected in a sample tube and the empty bag reweighed. The weight of the secreted fluid was given by the difference of the wet weight of the bag before and after incubation. The silk was removed and the tissue washed in distilled water and dried overnight at 110°.

RESULTS

Diffusion of H^+ ions through sheets of mucosa

Aerobic experiments. In these, a sheet of gastric mucosa separated a solution of HCl in stopper *S* from bicarbonate saline in the vessel *B* (see Fig. 1). In all experiments with non-secreting mucosa in contact with 0·1, 0·05 and 0·02 N-HCl solutions, evolution of CO_2 was observed while the volume of the HCl solution remained approximately constant, indicating that H^+ ions diffused across the membrane and liberated CO_2 from the bicarbonate solution. The output of CO_2 decreased gradually, and when the acidity of the remaining HCl solution was plotted against time, an exponential curve was obtained (Fig. 2). Titration of samples of the HCl solutions recovered after the experiments showed a reduction in the acidity which was equivalent to the amount of CO_2 evolved during the experiments.

The equation used by Teorell (1947)

$$H = H_0\, e^{-ct/p}$$

expresses the decrease in the acidity of the HCl solution with time, where H_0 = initial acidity of HCl solution (mmol. or μl.), H = acidity at time t (min.), p = volume of solution (ml.) and c = permeability coefficient (a constant). In nine aerobic experiments with 0·1 N-HCl, c for 1·3 cm.² sheets of gastric mucosa of average thickness 0·3 mm. was from

$3{\cdot}2 \times 10^{-4}$ to $13{\cdot}0 \times 10^{-4}$ ml. min.$^{-1}$ (average $9{\cdot}0 \times 10^{-4}$, standard deviation $2{\cdot}7 \times 10^{-4}$). Similarly, in seven experiments with 0·05, 0·02 and 0·01 N-HCl, c was from $2{\cdot}2 \times 10^{-4}$ to $11{\cdot}0 \times 10^{-4}$ ml. min.$^{-1}$ (average $7{\cdot}0 \times 10^{-4}$, standard deviation $2{\cdot}9 \times 10^{-4}$). There was, therefore, no significant difference between the values of c obtained with HCl solutions of different concentrations. Although all mucosae were tied to the base of stoppers of about 1·0 cm.^{2} cross-sectional area, they bulged to varying degrees, depending on the magnitude of the applied pressure and the state of contraction of the muscularis mucosae. This resulted in a slight variation in the surface areas of the mucosae, which, together with their different thicknesses (apart from individual variations the thickness of the mucosa also depends on the state of contraction), probably accounts for the relatively wide range of the values of c. In any one non-secreting mucosa, however, c was constant. This is illustrated in Fig. 2.

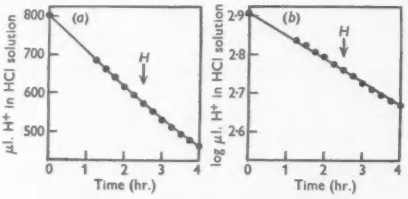

Fig. 2. Diffusion of H^+ ions through non-secreting gastric mucosa. Secretory side of mucosa in contact with 0·4 ml. 0·09 N-HCl. Incubated at 25·0° in bicarbonate saline containing 0·2% glucose, gassed with 5% CO_2+95% O_2. Histamine added from side arm at arrow marked *H* (final concentration 5×10^{-5} M). Pressure −20 mm. manometric fluid. Dry weight of secretory portion of mucosa 11·3 mg. In (*a*) the μl. H^+ remaining in the HCl solution, and in (*b*) log μl. H^+, are plotted against time.

The initial rate of CO_2 evolution depended only on the concentration of the HCl solution, and a comparison of results from experiments with 0·05, 0·02 and 0·01 N-HCl showed that the initial amounts of CO_2 evolved were approximately $\frac{1}{2}$, $\frac{1}{5}$ and $\frac{1}{10}$ respectively of the amount evolved from 0·1 N-HCl. The subsequent rate of CO_2 evolution followed the exponential equation.

The diffusion coefficient D (cm.^{2}sec.$^{-1}$) was calculated from c using the relation $c = D\frac{A}{h} \times 60$, where A = area of the membrane (cm.^{2}) and h = its thickness (cm.) (Teorell, 1947), or directly from the formula

$$D = \frac{h \times Q_{\text{ml.}}}{A \times t},$$

where $Q_{\text{ml.}}$ = the number of ml. of the HCl solution which contain an amount of H^+ ions equal to the amount which has diffused, and t = the time in sec. (see Bull, 1943). The average diffusion coefficient D for the above sixteen experiments was $0{\cdot}03 \times 10^{-5}$ cm.^{2}sec.$^{-1}$ (D for 0·1 N-HCl in aqueous solution at 19·3° is $2{\cdot}5 \times 10^{-5}$ cm.^{2}sec.$^{-1}$; Thovert, 1902).

The resultant pressures applied to the nutrient side of these mucosae varied from −40 to +20 mm. manometric fluid. Within this range the rate of diffusion of H^+ ions through aerobic mucosa was not dependent on the applied pressure.

Anaerobic experiments. When the pressure was equal on both sides of the mucosa (applied pressure = 0), the permeability coefficient c was of the same order in anaerobic experiments as aerobically. A complication arose when pressure was applied, since, under anaerobic conditions, gastric mucosa allows fluid to traverse it in the direction of applied pressure (Davies & Terner, 1949). On application of negative pressure some acid solution was forced through the tissue and the evolution of CO_2 from the bicarbonate of the medium was increased. The amount of CO_2 evolved by diffusing H^+ ions only was calculated by subtracting from the total amount of CO_2 evolved the equivalent of the amount of H^+ ions contained in the volume of solution which had been forced through the mucosa during the same period. This correction was made on the assumption that the fluid traversing the membrane contained the same concentration of H^+ ions as the HCl solution in contact with the tissue. The permeability coefficient c at negative pressures calculated in this way did not differ from the value determined for the same mucosa in the absence of applied pressure. Positive pressure forced neutral fluid from the nutrient to the secretory side; the rate of CO_2 evolution was reduced probably because the neutral fluid, which streamed through the mucosa in the direction opposing diffusion, diluted the HCl in contact with the tissue. This resulted in lower values for c.

Similar results were obtained in control experiments in which 'dead' frog gastric mucosa (tissue partly dried in air and then steeped in 0·01 M-KCN for 10 min.), dead pig bladder (dried tissue soaked in saline for several days before use) and sheets of cellophan were used as the membrane separating the HCl and bicarbonate solutions. For 'dead' mucosa the diffusion coefficient D was $0{\cdot}02 \times 10^{-5}$ cm.^{2}sec.$^{-1}$ (one experiment); for dead pig bladder D was $0{\cdot}15 \times 10^{-5}$ cm.^{2}sec.$^{-1}$.

Secretion and diffusion. Since HCl secretion by a gastric mucosa is accompanied by the uptake of an equivalent amount of CO_2 (Davies, 1948) it was expected that the output of CO_2, due to diffusion of H^+ ions from the secretory to the nutrient side of the tissue, would be reduced by the onset of secretion. Histamine was added from the side arm of the vessel after a steady evolution of CO_2 had been observed. Diminution of the rate of CO_2 evolution was not observed when 0·1 N-HCl was in contact with the

secretory side of the tissue. However, with 0·05N-HCl, stimulation by histamine resulted in a decrease of the CO_2 evolution. In Fig. 3 the reduction of CO_2 evolution during the period 1 hr. 45 min. to 2 hr. 45 min. (about 17 μl.) corresponds to an average Q_{HCl} of about 3. When thiocyanate, which inhibits secretion of HCl (Davenport, 1940; Crane, Davies & Longmuir, 1946), was added to a final concentration of 0·01M the original rate of CO_2 evolution was restored.

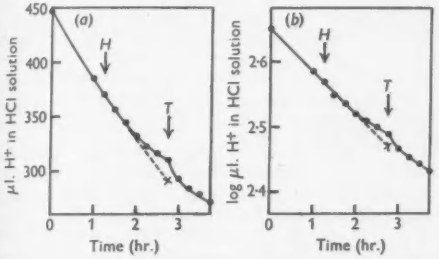

Fig. 3. Diffusion of H^+ ions through gastric mucosa. Effect of stimulation by histamine. Secretory side of mucosa in contact with 0·4 ml. 0·05N-HCl. Incubated at 25·0° in bicarbonate saline containing 0·2% glucose, gassed with 5% CO_2+95% O_2. In (*a*) the μl. H^+ in the HCl solution, and in (*b*) log μl. H^+, are plotted against time. Histamine added at arrow marked *H* (final concentration 5×10^{-5}M); thiocyanate added at arrow marked *T* (final concentration 0·01M). Pressure −30 mm. manometric fluid. Dry weight of secretory portion of mucosa 5·3 mg. The broken line indicates the (calculated) slope of the curve if there had been no secretory response to histamine.

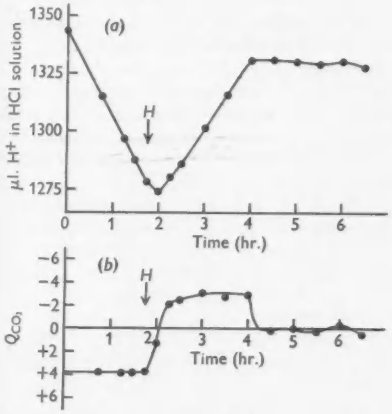

Fig. 4. Diffusion and secretion of H^+ ions. Secretory side of mucosa in contact with 3 ml. 0·02 N-HCl. Incubated at 25·0° in bicarbonate saline containing 0·2% glucose, gassed with 5% CO_2+95% O_2. In (*a*) the μl. H^+ in the HCl solution are plotted against time, in (*b*) the rates of diffusion and secretion of H^+ ions are given for comparison. Histamine added at arrow *H* (final concentration 5×10^{-5}M). Pressure −40 mm. manometric fluid. Dry weight of secretory portion of mucosa 10·3 mg.

When 0·02N-HCl was allowed to diffuse through a resting mucosa, the evolution of CO_2 stopped after stimulation by histamine, and the subsequent uptake of CO_2 showed that acid was being secreted by the tissue (Fig. 4). The acidity of the secreted HCl solution, calculated from the rate of fluid production and uptake of CO_2, was low. If, however, the average rate of CO_2 evolution ($q^{H^+}_{CO_2}$), observed before the addition of histamine, was added to the Q_{HCl}, the acidity of the secreted HCl solution was estimated to be between 0·1 and 0·12N (Table 1).

Table 1. *Back diffusion and secretion of* H^+ *ions*

(Secretory side of sheet of mucosa in contact with 0·5 ml. 0·02N-HCl, nutrient side with bicarbonate saline, gassed with 5% CO_2+95% O_2, and containing 0·2% glucose. Histamine added to nutrient saline from side arm at 0 hr. 45 min. Dry weight of secretory portion of mucosa 7·0 mg. Pressure −10 mm. manometric fluid. $+Q_{CO_2}=q^{H^+}_{CO_2}$; $-Q_{CO_2}=Q_{HCl}$.)

Time (hr.)	Observed (μl./mg. dry wt./hr.) Q_{CO_2}	q_{H_2O}	Apparent molarity of secreted HCl	$Q_{HCl}+q^{H^+}_{CO_2}$ (μl./mg. dry wt./hr.)	Calculated molarity of secreted HCl
1	+2·4	0	—	—	—
2	−1·6	1·3	0·06	4·0	0·14
3	−2·2	1·7	0·06	4·6	0·12
4	−1·6	1·6	0·045	4·0	0·11
5	−0·9	1·3	0·03	3·3	0·11

Addition of HCl *solutions to secreting mucosa.* In the experiments described above, solutions of HCl were placed in contact with the secretory side of freshly dissected gastric mucosa. Although the mucosae were undoubtedly alive, the diffusion rates were high and often exceeded the fastest secretory rate so far observed ($Q_{HCl}=10$), and it seemed probable that the tissue had been damaged. In modified experiments isotonic sodium chloride solution was placed in contact with the secretory side of the mucosa; the latter was incubated with bicarbonate saline, and secretion was induced by histamine. When the secretory response was established, as shown by the uptake of CO_2, the apparatus was opened and strong HCl added to the NaCl in contact with the mucosa to give a 0·1 or 0·12N-HCl solution. The acid was thus added after the mucosa had recovered from the trauma due to manipulation and had covered itself with mucus. The uptake of CO_2 continued for varying periods at a reduced rate and was then followed by evolution of CO_2. In these experiments the diffusion coefficient *D* was from $0{\cdot}01 \times 10^{-5}$ to $0{\cdot}015 \times 10^{-5}$ cm.^{2}sec.$^{-1}$.

Table 2. *Back diffusion of secreted* H^+ *ions*

(Tied mucosae were incubated in bicarbonate saline containing 0·2 % glucose, gassed with 5 % CO_2 + 95 % O_2. Histamine was added to a concentration of 5×10^{-5} M. At the end of the experiments on mucosae *D* and *E*, thiocyanate was added to a concentration of 0·02 M. The tubes were weighed (*a*) before, (*b*) after incubation, and (*c*) after emptying. The difference (*b*) − (*a*) = weight of secretion, (*b*) − (*c*) = weight of contents. The difference between the weight of contents and secretion corresponds to the difference in the weight of the empty tissue before and after incubation, and is mainly due to extrusion of mucus and some tissue degradation. The high pH values observed at the end of the experiments are partly due to buffering of the HCl by cellular debris.)

Mucosa	Dry wt. of mucosa (mg.)	Area of mucosa ($cm.^2$)	Wet wt. of contents (mg.)	Wet wt. of secretion (mg.)	Duration of secretory period (hr.)	H^+ produced (μl.)	Molarity of H^+ in secretion at end of secretory period	CO_2 evolved (μl.)	Duration of back diffusion (hr.)	pH of contents at end of exp.	Average (μl./mg. dry wt./hr.) Q_{HCl}	q_{H_2O}	$q^{H^+}_{CO_2}$	State of tissue
A	43·2	5·64	667	590	4·0	828	0·063	232	3	2·9	4·8	3·4	1·8	Undamaged
B	15·0	2·54	—	—	3·5	286	—	57 (+66 leak)	1	2·6	5·4	—	3·1	1 perforation, 2 small ulcers at 6·5 hr.
C	12·6	1·85	—	—	4·0	242	—	(54 leak)	—	1·75	4·8	—	—	1 perforation at 5 hr.
D	41·0	5·25	456·5	430	9·25	650	0·068	180	1	1·84	1·7	1·1	—	Undamaged
E	39·0	5·45	215	185	8·5	216	0·052	36	—	3·48	0·7	0·6	—	Undamaged

Experiments in phosphate saline. With phosphate saline, gassed with 100 % O_2, as the nutrient medium, the rate of diffusion of H^+ ions could not be observed manometrically. The overall reduction in acidity was determined by titration of samples of the HCl solution recovered after incubation, and it was of the same order as in corresponding experiments in bicarbonate saline. Stimulation by histamine resulted in an increase of the Q_{O_2} (see Davies, 1948). In two experiments both mucosae, which were in contact with 0·1 N-HCl, showed signs of damage 0·5 and 1 hr. respectively after their Q_{O_2} values had increased; their Q_{O_2} fell to about 50 % of its maximum value and the mucosae leaked. One mucosa, in contact with 0·1 N-HCl to which histamine was not added, had an average Q_{O_2} of −1·5 throughout a 3·5 hr. experiment and appeared undamaged. In the presence of more dilute HCl solutions, the Q_{O_2} increased after stimulation by histamine and remained steady during experiments of 3–4 hr. duration without damage to the tissue.

Diffusion of secreted H^+ *ions through tied mucosa*

Tied gastric mucosae were incubated in the bicarbonate saline, gassed with 5 % CO_2 + 95 % O_2, in Warburg cups and stimulated by addition of histamine (Davies, 1948). The experiments were extended over 10 hr. since it was expected that some of the acid accumulated in the bags might diffuse out at the end of the secretory phase. Typical results are given in Tables 2 and 3. Evolution of CO_2 due to back diffusion of H^+ ions was observed in three out of ten experiments. Four highly active mucosae (max. $Q_{HCl} > 5$) were seen to have perforated ulcers when CO_2 evolution started (Davies & Longmuir, 1948, reported and discussed one case of ulcer formation in bicarbonate saline). However, mucosae with even larger perforations could continue to take up CO_2, unless some of the contents were forced out by the pressure in the bag (Table 3, mucosa *C*). This may be due to buffering of the acid contents by cell debris from the damaged area, with a consequent reduction of the rate of back diffusion. On the other hand, mucosae of low secretory activity could maintain a steady rate of CO_2 uptake for many hours with no observable back diffusion (Table 3, *D* and *E*).

Table 3. *Time course of gas exchanges of experiments summarized in Table 2*

Time (hr. min.)	Q_{CO_2} *A*	*B*	*C*	*D*	*E*
0 00					
0 30	Histamine added from side arm			Histamine in nutrient medium	
1 00	−5·3	—	−4·8	−2·1	−0·3
1 30	−6·5	−5·3	−5·6	−1·9	−0·7
2 00	−6·5	−6·9	−5·4	−1·8	−0·8
2 30	−6·3	−7·2	−5·9	−2·0	−1·0
3 00	−5·9	−6·4	−4·9	−1·9	−1·5
3 30	−4·9	−6·0	−4·9	−2·0	−1·0
4 00	−2·7	−4·1	−3·8	−1·9	−1·1
4 30	−0·2	−2·3	−2·3	−2·0	−0·7
5 00	+1·9	0	+4·9*	−1·7	−0·9
5 30	+3·2	+2·8	+2·6	−2·2	−0·4
6 00	+3·0	+3·4	+1·0	−1·7	−0·7
6 30	+1·9	+8·8*	−0·8	−1·9	−0·6
7 00	+0·7	Open sheet	−1·6	−1·9	−0·4
7 30	+0·9	—	−0·3	−1·4	−0·3
8 00	−0·5	−0·9	−0·9	−1·1	−0·4
8 30	−0·7	−0·5	+0·3	−1·2	−0·4
9 00	—	Sheet transferred to phosphate saline gassed with 100 % O_2	+0·7	−0·7	−0·3†
9 15	—		—	−0·4†	+1·0
9 30	—	Q_{O_2}	—	+2·9	+1·0
9 45	—	−2·7	—	+4·9	+1·0
10 00	—	−2·4	—	+5·1	+0·9
10 15	—	−1·0	—	+4·7	—

* Perforation observed.

† Thiocyanate added from side arm (final concentration 0·02 M).

When HCl was secreted at rapid rates the secretory phase was followed by a period during which acid diffused out and liberated CO_2 from the bicarbonate in the medium (Table 3, *A* and *B*). Perforation of mucosa *B* resulted in increased evolution of CO_2 due to extrusion of some acid contents. In order to decide whether the evolution of CO_2 was due to diffusion or glycolysis, the following experiment was carried out. Two mucosae were removed from their cups, emptied, washed in saline and the open sheets replaced in bicarbonate saline, gassed with 5 % CO_2 + 95 % O_2. Readings were continued, and a small uptake of gas showed that the previously observed evolution of CO_2 was not due to aerobic glycolysis. The tissues were then transferred to cups containing phosphate saline, gassed with 100 % O_2, and incubated for a further 1·5 hr. Their average Q_{O_2} during this period was −2·0 (see Table 3, *B*).

In one very large mucosa (Table 3, *A*) the rate of back diffusion of H^+ ions reached a peak between 5·5 and 6 hr., and then decreased. At 8 hr. CO_2 evolution stopped and a small uptake of gas of the same magnitude, as with open tissue, showed that the tissue was still alive and now in the resting state. On removal from the cup this mucosa was inspected; the bag was only about half filled, undamaged, and showed no signs of perforation even on squeezing. It contained a clear watery fluid admixed with particles of opaque material, apparently derived from cell debris. Buffering by this cell material and the loss of H^+ ions by diffusion most probably account for the relatively high pH observed (2·9).

The molarity of the HCl solution in the secretion at the end of the secretory period (i.e. before back diffusion started) was calculated (*a*) from the amount of extra CO_2 taken up (equivalent to the amount of HCl produced (Davies, 1948)), which was measured manometrically, and the volume of the secretion (increase of the wet weight of the tied mucosa); and (*b*) by titration of the H^+ in the gastric contents recovered after incubation, and correction for the amount of H^+ lost by diffusion (amount of CO_2 evolved in the manometric experiment), and for the difference between the wet weights of contents and secretion.

It should be noted that the pH values given in Table 2 were determined after the completion of the experiments. They do not, therefore, represent the pH of the gastric contents during the experiments, although they give some indication of the relation between the pH and the rate of diffusion. Davies & Longmuir (1948) found that the pH of the contents of tied mucosa, incubated in bicarbonate saline for short periods, can be close to 1; the highest concentration of H^+ in gastric secretion reported by these authors was 0·109 M. However, in experiments of longer duration, H^+ ion concentrations of less than 0·06 M were observed by Davies (1948).

Inhibition of secretion. Addition of an inhibitor, thiocyanate, resulted in evolution of CO_2 due to back diffusion of H^+ ions, when the pH of the gastric contents was less than about 2. At the end of experiments on mucosae *D* and *E*, thiocyanate was added to a final concentration of 0·02 M. The evolution of CO_2 which followed immediately was small in *E*, and was possibly due to aerobic glycolysis, since the pH of the contents, 3·48, was too high for diffusion to occur at a measurable rate, if at all. Mucosa *D*, however, showed a large evolution of CO_2 which was probably due to the combined effect of diffusion and glycolysis. The pH of its contents, measured after the experiment, was 1·84.

Diluting secretions. Since most workers emphasize the importance of dilution and neutralization of the secreted HCl by mucus (see Babkin, 1944; Hollander, 1938), attempts were made to estimate the neutralizing power of the mucus in frog stomachs. Eugenol, which has been shown to stimulate secretion of mucus in dogs (Hollander & Lauber, 1948), was used to increase the production of mucus by isolated frog gastric mucosa. Five tied mucosae were injected with 0·03–0·1 ml. of an approximately 1 % emulsion of eugenol in water (stabilized by 50 mg. of cetyl alcohol containing ethylene oxide/100 ml.), and placed in bicarbonate or phosphate saline in Warburg cups. After 1 hr. incubation at 25·0°, the contents of the bags were removed, weighed and titrated against 0·01 N-HCl, in an atmosphere of 5 % CO_2 + 95 % O_2. All the mucosae contained a viscous, opaque mucous material of pH 5·8–7·5. The titration curves showed that the samples were buffered between their initial pH and pH 3. In this pH range, their neutralizing capacity corresponded to that of 0·03–0·04 N-solutions of pure alkali.

Table 4. *Diffusion coefficients of* HCl

Tissue	$D \times 10^5$ (cm.^{2}sec.$^{-1}$)
—	2·5*
Dead pig bladder	0·15
Anaerobic frog gastric mucosa	0·04
'Dead' frog gastric mucosa	0·02
Frog gastric mucosa (added HCl)	0·03
Frog gastric mucosa (secreted HCl)	0·01–0·016

* In aqueous solution at 19·3° (Thovert, 1902).

DISCUSSION

Evidence in support of the diffusion theory. Teorell's conclusions that the reduction of the acidity of the gastric juice is mainly due to the diffusion of H^+ ions through the gastric mucosa into the blood (Teorell, 1933) have been criticized by other workers (Hollander, 1938), particularly because of the conditions of the experiments, since the volume of the instilled HCl solutions had to be small in relation to the size

of the stomach in order that the change in acidity might be sufficiently large to be detected by analysis (Babkin, 1944). As a result, the diffusion theory does not appear to have found general acceptance.

In the experiments described in this paper relatively large volumes of HCl solution were placed in contact with the secretory sides of sheets of gastric mucosa, and the rate of diffusion of H^+ ions was followed by manometric measurement of the amount of CO_2 liberated from the bicarbonate solution on the nutrient side. With non-secreting mucosa the rate of diffusion depended on the concentration of the HCl solution, and consequently decreased exponentially with time, in agreement with the diffusion theory (Teorell, 1947). The onset of secretion of HCl by the mucosa after histamine stimulation could reduce the output of CO_2 due to diffusion (Fig. 3), or result in a net uptake of CO_2 (Fig. 4). Since, under the conditions of the experiment, secretion of HCl is accompanied by an uptake of CO_2 equivalent to the amount of HCl produced (Davies, 1948), these results suggest that secretion and back diffusion can occur at the same time, and that the observed uptake or output of CO_2 is the balance of the amount of CO_2 taken up during secretion of HCl by the mucosa and the amount evolved by back diffusion of H^+ ions. This is supported also by the observation that the molarity of the HCl solution secreted by mucosae, whose secretory sides were in contact with HCl solutions, was low. If, however, on the assumption that secretion and back diffusion of H^+ ions can occur at the same time, the true Q_{HCl} was taken as the sum of the observed Q_{HCl} and the $q^{H^+}_{CO_2}$, then the calculated molarity was between 0·10 and 0·12N (Table 1). This, allowing for some dilution by mucus, is in agreement with the generally accepted view that the HCl solution secreted by the gastric glands is isotonic with the blood (Babkin, 1944).

The relation of back diffusion to secretion. In experiments in which HCl solutions were placed in contact with sheets of gastric mucosa, the diffusion rates were high ($D = 0{\cdot}03 \times 10^{-5}$ cm.^{2}sec.$^{-1}$ corresponds to a $q^{H^+}_{CO_2}$ of 12 with 0·1N-HCl, which is of the order of the highest Q_{HCl} observed, and of 2·4 with 0·2N-HCl, for a mucosa of 1·3 cm.^{2} secretory area and 8 mg. dry weight). Although mucosae in contact with 0·1N-HCl did not respond to histamine stimulation, it was at least possible to observe secretory responses when lower concentrations of HCl (0·05 and 0·02N) were used. Lower diffusion rates ($D = 0{\cdot}01$–$0{\cdot}015 \times 10^{-5}$ cm.^{2}sec.$^{-1}$) were observed when 0·1N-HCl was added to secreting tissue, or when the acid secreted by a tied mucosa was diffusing out at the end of the secretory phase. The reason for this difference is not clear, but it is possible that the tissue suffered some damage when HCl solutions were placed in contact with it before it had recovered from injury due to dissection and manipulation. (For comparison of diffusion coefficients see Table 4.)

The relative contributions of dilution, neutralization and diffusion to the reduction of gastric acidity. If it is accepted that the primary acidity of the secreted HCl is 0·12N in the frog (isotonic with blood), an explanation is required for the low concentration of HCl in the secretion of tied mucosa at the end of the secretory phase. In the experiments in Table 2 the deficit of H^+ ions ranged from 43 to 56% (cf. Davies, 1948, Table 4). Possible explanations for the reduction of acidity are (*a*) dilution by a neutral secretion, (*b*) dilution and neutralization by a secretion of great neutralizing power, (*c*) loss of H^+ ions by back diffusion, and (*d*) a combination of (*c*) with (*a*) or (*b*).

In mucosa *A* the concentration of HCl in the secretion at the end of the secretory phase was 0·063N. This could correspond to a loss of 47% of the H^+ ions or to secretion of the corresponding amount of a neutral fluid. If it is assumed that back diffusion occurred while the HCl was being secreted, then the true rate of CO_2 uptake corresponding to the HCl produced was the sum of the observed Q_{HCl} and the $q^{H^+}_{CO_2}$. The value of the latter during active secretion could not be determined, but it can be assumed to be at least equal to the maximum $q^{H^+}_{CO_2}$ observed at a later stage. Thus the sum of the average Q_{HCl} (4·8) and the maximum $q^{H^+}_{CO_2}$ (3·2) would give the true average Q_{HCl} (8·0), and the primary acidity of the secreted HCl solution calculated from this and the average q_{H_2O} (3·4) would be 0·105N. Some reduction of the acidity may be due to neutralization by mucus. An estimate of the amount of mucus extruded from the mucous cells may be obtained from the difference between the wet weight of the contents and the secretion (cf. Davies, 1948), or from the difference between the wet weights of a mucosa before and after incubation, although this may include some cell debris. It seems reasonable to assume that only the mucus, present as such or as a precursor in the mucous cells at the beginning of the experiment, is extruded into the lumen, and that there is no regeneration of mucus by an isolated mucosa, incubated in an inorganic medium containing glucose as the only added substrate. The neutralizing capacity of gastric mucus in frogs was found to be equivalent to that of a solution of about 0·05N-alkali (see p. 155). Since the difference between the initial and final wet weight (or between the wet weights of contents and secretion) of mucosa *A* was about 80 mg. (Table 2), this amount, if equivalent to 0·05N-alkali, could have neutralized about 90 μl. of the total 1600 μl. H^+ (590 mg. secretion containing 0·12N-HCl is equivalent to 1600 μl. H^+). Since the mucosa contained 830 μl. H^+ at the end of the secretory period of 4 hr., 680 μl. H^+ are left to be accounted for by diffusion. This amount corresponds to a $q^{H^+}_{CO_2}$ of 4·0, which is

greater than the maximum $q^{H^+}_{CO_2}$ observed. However, the neutralizing capacity of the mucus may have been greater than assumed in this calculation (but even if equivalent to 0·12N-alkali, it could not have neutralized more than 200 μl. H^+), and the rate of diffusion may have been higher during the period of active secretion than several hours later, when the H^+ ion concentration was already reduced. It was also assumed that there was no appreciable amount of other diluting secretions. This seems to be justified on histological grounds, since in frog gastric mucosa, apart from the oxyphilic cells of the chief glands, all other cells of glands and surface epithelium are of mucoid character (Pernkopf & Lehner, 1937). It is therefore very probable that in the very active mucosa *A* the reduction of acidity during the secretory phase was mainly due to back diffusion, and that this was so to a large extent also in mucosae *D* and *E*, although with slow secretory rates, dilution and neutralization may be of relatively greater importance than with faster rates.

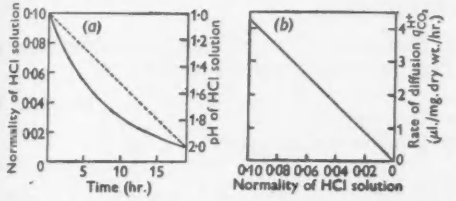

Fig. 5. Diffusion of H^+ ions through non-secreting gastric mucosa. Data calculated for frog gastric mucosa, area 1 cm.², thickness 0·3 mm., dry weight 6·0 mg., in contact with 0·1 ml. pure unbuffered HCl solution; $c = 2 \times 10^{-4}$ ml. min.$^{-1}$, $D = 0{\cdot}01 \times 10^{-5}$ cm.²sec.$^{-1}$. (*a*), unbroken line, decrease of normality of HCl with time; broken line, increase of pH with time; (*b*), relation of rate of diffusion to normality of HCl solution.

The relation between rate of back diffusion and H^+ *ion concentration of the gastric contents.* Since the concentration of an HCl solution, in contact with a membrane through which it can diffuse, decreases exponentially with time, the rate of diffusion ($q^{H^+}_{CO_2}$) depends on the prevailing H^+ ion concentration (Teorell, 1933), of which it is a linear function. The H^+ ion concentration must be high (pH less than about 2) for diffusion to be fast enough to be measurable (see Fig. 5, which has been constructed for an ideal non-secreting mucosa in contact with pure, unbuffered HCl solution). This may explain why back diffusion was observed in highly active mucosa, but not in mucosa of low secretory activity. With fast rates of secretion, high concentrations of H^+ ions are built up rapidly so that, when the rate of secretion decreases towards the end of the secretory phase, it is outstripped by the rate of back diffusion. With low secretory rates the decrease in the H^+ ion concentration due to dilution, buffering by mucus and cell material and to diffusion results in a reduction of the rate of back diffusion, so that it cannot overtake the rate of secretion which can be maintained at a low, but steady level for many hours. Since the observed CO_2 uptake is the resultant of the CO_2 uptake due to HCl secretion and the CO_2 output due to back diffusion, inhibition of secretion should result in the evolution of CO_2, due to diffusion only, provided the H^+ ion concentration of the gastric contents is sufficiently high (Table 2, *D*).

The experiments described in this paper show that H^+ ions can diffuse through living isolated gastric mucosa and support Teorell's diffusion theory (Teorell, 1933, 1947). They suggest that back diffusion of H^+ ions also occurs during secretion of HCl, thus reducing the efficiency of the process. In the frog, the loss of H^+ ions by back diffusion during the secretory phase can be relatively large; in the mammal, however, owing to the greater thickness of the mucosa and the high secretory rates (ten times as fast as in the frog) the loss of H^+ ions may be comparatively small.

SUMMARY

1. The diffusion of H^+ ions through isolated gastric mucosa of frogs and toads was studied by gasometric methods.

2. Solutions of HCl were placed in contact with the secretory side of sheets of gastric mucosa, which were incubated in bicarbonate saline. With non-secreting tissue an output of CO_2 was observed which decreased exponentially with time, in agreement with the findings of Teorell (1933, 1947).

3. On stimulation of mucosa in contact with HCl by 5×10^{-5}M-histamine, the evolution of CO_2 ceased, and the subsequent rates of CO_2 uptake and fluid secretion corresponded to the production of HCl solutions of low concentration. If, however, it was assumed that the rate of CO_2 uptake had been reduced by simultaneous evolution of CO_2 due to back diffusion of H^+ ions, the calculated concentration of the secreted HCl solution was near isotonicity.

4. In prolonged experiments with highly active, tied gastric mucosa incubated in bicarbonate saline, back diffusion of H^+ ions was observed at the end of the secretory phase. Mucosa of low secretory activity, however, continued to take up CO_2 for at least 10 hr. Inhibition of secretion by 0·02M-thiocyanate was followed by back diffusion when the pH of the gastric contents was less than 2.

5. At the end of the secretory phase the concentration of HCl in the secretion was 40–60 % of the theoretical 0·12N. The loss can be largely accounted for by back diffusion of H^+ ions during the secretion of HCl.

6. The results show that back diffusion of H^+ ions can occur in living isolated frog gastric mucosa, and that the rate of diffusion is linearly related to the H^+ ion concentration of the gastric contents. They suggest that the concentration of HCl in the secretion is reduced by back diffusion not only after the cessation of secretory activity, but also during the period of HCl production.

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Studies in Detoxication

26. THE FATES OF PHENOL, PHENYLSULPHURIC ACID AND PHENYLGLUCURONIDE IN THE RABBIT, IN RELATION TO THE METABOLISM OF BENZENE

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From studies of the fate of benzene (Porteous & Williams, 1949*a*, *b*), catechol (Garton & Williams, 1948), resorcinol and quinol (Garton & Williams, 1949) in the rabbit, the following scheme for the oxidation of benzene was suggested:

Benzene → phenol → catechol → hydroxyquinol.
↓
quinol

In order to obtain further evidence about this scheme and to find out whether phenol conjugates were oxidized, the metabolic fates of phenol, phenylsulphuric acid and phenylglucuronide were studied in detail.

Porteous & Williams (1949*a*) showed that when phenol is fed orally to rabbits practically all the phenol excreted is conjugated. The conjugated phenol excreted accounted for 80–90% of the phenol fed, although the total conjugation (i.e. glucuronic acid and ethereal sulphate) accounted for 100%. The difference between the total conjugation and conjugated phenol, amounting to some 10%, was assumed to be oxidation products of phenol. The nature of these oxidation products is described here.

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Quinol is known, by isolation, to be an oxidation product of phenol in the dog (Baumann & Preusse, 1879*a*, *b*). Brieger (1879) claims to have isolated catechol and quinol from 40 l. of urine obtained from hospital patients treated with phenol, but no reference is made to the quantities isolated or to the amount of phenol administered.

Phenylsulphuric acid is well known to be a metabolite of phenol. On feeding the compound to rabbits, 80–95% can be accounted for in the urine as ethereal sulphate (Rhode, 1923; see also Auerbach, 1879 and Christiani, 1878). Baumann & Preusse (1879*b*) reported that after feeding the potassium salt to a dog, quinol but not catechol could be detected in the hydrolysed urine. Furthermore, Sperber (1948*b*) has shown that, on injection of the sodium or potassium salts of phenylsulphuric acid into hens, some 75% can be recovered in the urine in 80 min. and there is no rise in the glucuronide output. These results indicate that phenylsulphuric acid is rapidly eliminated from the body mainly as such, but a small proportion may be oxidized to quinol.

The other well-known metabolite of phenol is phenylglucuronide. Nakano (1937) found that this compound, on injection, is excreted almost quantita-

tively unchanged, but, when fed, only about a half is excreted in the urine and there is a rise in the ethereal sulphate output. Sperber (1948*b*), however, found that when injected into hens 57 % of the dose could be recovered in the urine as glucuronide. There are no reports of phenylglucuronide giving rise to oxidation products.

The study of these two conjugates was undertaken because we wanted to find out whether free phenol or conjugated phenol was the precursor of the polyphenols of benzene urine.

EXPERIMENTAL AND RESULTS

I. *Excretion of ethereal sulphate and of glucuronide after administration of phenylsulphate or phenylglucuronide*

Glucuronic acid was determined colorimetrically (Hanson, Mills & Williams, 1944) and ethereal sulphate gravimetrically by Folin's method (cf. Williams, 1938).

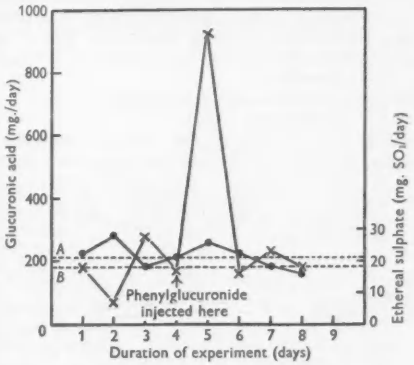


Fig. 1. The effect of injecting phenylglucuronide (1·21 g.) on the ethereal sulphate and glucuronic acid outputs of rabbit no. 130; ●—●, ethereal sulphate; ×—×, glucuronic acid. The dotted lines show the average normal output, *A* of ethereal sulphate and *B* of glucuronic acid.

The potassium phenylsulphate used was prepared according to Burkhardt & Lapworth (1926) and phenylglucuronide dihydrate according to Porteous & Williams (1949*a*) and Garton, Robinson & Williams (1949). The constants for these compounds are as recorded by Garton *et al.* (1949).

The rabbits used were kept on a diet of Lever cubes (50 g./day) and water *ad lib.* during experimental periods. This diet appeared to cause the least fluctuations in the normal ethereal sulphate and glucuronic acid outputs.

For feeding experiments potassium phenylsulphate and phenylglucuronide dihydrate were dissolved in water. For the injection experiments 2·42 g. of phenylglucuronide dihydrate were dissolved in 40 ml. water. This solution was maintained at 30° and 10 ml. injected into the loose skin of the groin at each side, each animal thus receiving 20 ml. (i.e. 1·21 g. of the glucuronide).

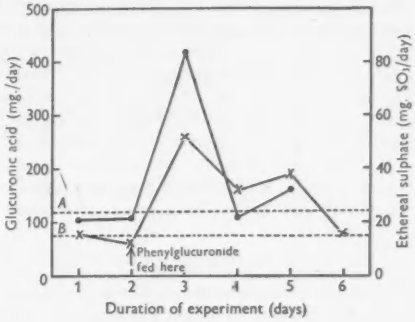


Fig. 2. The effect of oral administration of phenylglucuronide (1·5 g.) on the ethereal sulphate and glucuronic acid outputs of rabbit no. 124; ●—●, ethereal sulphate; ×—×, glucuronic acid. The dotted lines show the average normal output, *A* of ethereal sulphate, *B* of glucuronic acid.

The results are recorded in Table 1, which shows that when potassium phenylsulphate is fed some 90 % of it can be accounted for as 'extra' ethereal sulphate. The glucuronic acid output does not appear to be

Table 1. *The excretion of ethereal sulphate and glucuronic acid by rabbits receiving potassium phenylsulphate or phenylglucuronide*

Compound and route of administration	Rabbit no.	Dose g.	Dose mg./kg.	'Extra' ethereal sulphate (% of dose)	'Extra' glucuronic acid (% of dose)	Total recovered (% of dose)
Potassium phenylsulphate:						
Oral	124	0·33	100	82·9	0	82·9
Oral	125	0·36	100	90·3	0	90·3
Oral	126	0·36	100	89·0	0	89·0
Oral	124	1·65	500	—	0	—
Oral	125	1·80	500	—	0	—
Phenylglucuronide:						
Oral	124	1·40	500	21·3	28·4	49·7
Oral	125	1·40	500	15·4	41·5	56·9
Subcutaneous injection	129	1·21	403	0	98·5	98·5
Subcutaneous injection	130	1·21	378	0	96·2	96·2

disturbed. At a dose level of 100 mg. (≡44 mg. phenol)/kg. body weight a small rise in glucuronic acid cannot be readily detected by the quantitative method used. Two determinations of glucuronic acid output were therefore made at the higher level of 500 mg. (≡222 mg. phenol)/kg., but again no disturbance of the normal glucuronic acid level could be detected. It is concluded, therefore, that very little potassium phenylsulphate is hydrolysed in the intestine or in the body. If hydrolysis had been extensive then the excretion of phenylglucuronide would have been expected to rise.

Table 1 shows clearly that the fate of phenylglucuronide depends on the route of administration. On injection the glucuronide is almost quantitatively recovered in the urine in 1 day (see Fig. 1). On oral administration, however, there is an appreciable rise in the ethereal sulphate output and only about 50% of the glucuronide fed can be accounted for (i.e. by the 'extra' ethereal sulphate and glucuronic acid). The excretion of 'extra' glucuronic acid continues for 3 days after feeding, although the excretion of 'extra' sulphate is complete in 1 day (see Fig. 2). That there is a prolonged excretion of glucuronide after feeding phenylglucuronide is supported by qualitative findings reported below.

II. *Isolation of the oxidation products of phenol*

(1) *Preparation of extracts.* Twelve rabbits were each given twice a day on two successive days 0·25 g. phenol in 10 ml. water by stomach tube (total fed, 12 g.). On the first day 1975 ml. urine were collected and on the second 2000 ml. The slightly alkaline urine was non-reducing and a little darker than normal. It gave an intense Tollens test, but no $FeCl_3$ test though small amounts of free phenol were detected with 2:6-dichloroquinone-chloroimide. No other free phenols were detected. Each day's urine was filtered, made normal with respect to HCl and then heated for 0·5 hr. at 94° on a water bath to hydrolyse ethereal sulphates (cf. Garton *et al.* 1949). The urines were then extracted to exhaustion (30 hr.) with peroxide-free ether. Two extracts, A_E (for day 1) and B_E (for day 2) were thus obtained.

The extracted urines were now made 3 N with respect to HCl, boiled under reflux for 2 hr. to hydrolyse glucuronides and again extracted with ether for 30 hr. Two further extracts A_G and B_G were thus obtained.

The four extracts A_E, B_E, A_G and B_G were evaporated at 20° to about 20 ml. and examined for phenols by colour tests as described by Porteous & Williams (1949*b*). A_E and B_E were found to contain phenol, catechol, quinol and hydroxyquinol. A_G and B_G gave strong tests for phenol and very weak tests for catechol. None of the extracts contained resorcinol, pyrogallol or phloroglucinol. It was clear from these tests that the oxidation products of phenol were almost entirely in the ethereal sulphate fraction.

(2) *Isolation of phenols from the ethereal sulphate fraction.* Extracts A_E and B_E were combined, 30 ml. water added and the ether removed at 20°. The yellow-brown aqueous residue was boiled with charcoal and filtered. The cooled filtrate was made just alkaline with $NaHCO_3$ and treated with saturated basic lead acetate until precipitation was complete. The filtrate was freed of Pb with H_2S and extracted with ether for 20 hr. Evaporation of the ether at 20° in the presence of 10 ml. water gave an aqueous residue which was then treated with 50 ml. saturated $NaHCO_3$ and 20 ml. acetone containing 1 g. *p*-toluenesulphonyl chloride. After shaking the mixture for an hour and then keeping overnight, the whole was diluted with 150 ml. water. A crystalline product (980 mg., m.p. 80–90°) was obtained, which on fractional crystallization from absolute ethanol (charcoal) yielded (in order of crystallization) (*a*) quinol di-*p*-toluenesulphonate (50 mg., m.p. 155°); on recrystallization it had m.p. and mixed m.p. 158° with authentic material, but depressed the m.p. of catechol di-*p*-toluenesulphonate; (*b*) catechol di-*p*-toluenesulphonate (15 mg.), m.p. 160° and mixed m.p. 160°; it depressed the m.p. of the quinol ester; (*c*) phenol *p*-toluenesulphonate (675 mg.), m.p. and mixed m.p. 91–92°. We were unable to isolate the hydroxyquinol derivative, although colour reactions indicated very definitely that hydroxyquinol was present in the ethereal sulphate fraction.

The basic lead acetate precipitate was suspended in water and the lead removed with H_2S. The lead-free filtrate was treated in the same way as above (yield of toluenesulphonates, 2·27 g.) resulting in the isolation of quinol di-*p*-toluenesulphonate (136 mg.), m.p. and mixed m.p. 158–159°, and phenol *p*-toluenesulphonate (2 g.), m.p. and mixed m.p. 92°, each after recrystallization.

The yields of the phenols are summarized in Table 2.

Table 2. *Phenols isolated from the ethereal sulphate fraction of the urine of rabbits receiving phenol orally*

Compound isolated	Yield of *p*-toluenesulphonic ester (g.)	Yield as percentage of dose of phenol
Phenol	2·67	8·5
Quinol	0·186	0·41
Catechol	0·015	0·033
Hydroxyquinol	—	Small amounts detected by colour reactions

(3) *Isolation of phenol from the glucuronide fraction.* The only hydroxybenzene which appeared to be present in quantity in extracts A_G and B_G was phenol itself. Traces of catechol were detected by colour reactions. Treatment of the combined ether-free fractions A_G and B_G with *p*-toluenesulphonyl chloride in acetone yielded 3·15 g. of phenol *p*-toluenesulphonate (m.p. and mixed m.p. 92° after recrystallization) corresponding to 10% of the dose. This low yield of phenol indicates either that the glucuronide fraction was incompletely hydrolysed or that phenol is lost in the more vigorous hydrolysis by combination with other urinary constituents. It was therefore decided to investigate the glucuronide fraction obtained directly from phenol urine by systematic lead acetate precipitation.

(4) *Examination of the basic lead acetate fraction of phenol urine.* Phenol (0·8 g.) was fed to each of six rabbits and an 18 hr. urine (750 ml.) collected. The basic lead acetate precipitate was prepared as for the preparation of phenylglucuronide (Porteous & Williams, 1949*a*) and the Pb removed by H_2S. From the Pb-free filtrate 4·23 g. of phenylglucuronide dihydrate were isolated. This corresponds to 31% of the dose. The concentrated mother liquors (16 ml.) from the glucuronide were now examined for polyphenols.

They were hydrolysed by refluxing for 1 hr. with 6 ml. conc. H_2SO_4. The hydrolysate was cooled and diluted with water to 50 ml. and then extracted with peroxide-free ether for 8·5 hr. The extract thus obtained was tested for phenols, and it was found that only phenol itself was present, although doubtful tests for quinol were obtained. It was therefore concluded that only phenol occurs in the glucuronide fraction of phenol urines. The traces of catechol and quinol were insignificant.

Urines were collected for a further 3 days after the urines examined above. These urines appeared to be normal. They were, however, subjected to the same procedures as described above for the urines of the first 2 days. The extracts obtained did not contain significant amounts of phenolic metabolites and we detected traces of phenol and catechol in the ethereal sulphate fractions only. These traces were no more than can be found in similarly treated extracts of normal rabbit urine.

III. *The metabolites of potassium phenylsulphate*

(1) *Examination of the ethereal sulphate fraction of phenylsulphuric acid urine.* Ten rabbits were each given 2 g. of potassium phenylsulphate in water by stomach tube and a 24 hr. urine (950 ml.) collected, and filtered through glass wool. The non-reducing urine was made normal with respect to HCl and hydrolysed by warming to 94° for 30 min. The liberated phenols were then extracted (20 hr.) with peroxide-free ether. After adding 100 ml. water, the ether was removed *in vacuo* at 20°. The residual liquor, which contained globules of dark red oil, was boiled with charcoal and filtered. The filtrate contained much phenol, small amounts of quinol and only slight traces of catechol and hydroxyquinol.

Since the amounts of quinol present were small, it was decided to estimate it on a sample of the solution before attempting isolation. The quinol was estimated iodometrically as described in an earlier paper (Garton & Williams, 1949) by the method of Wieland (1910). It was found that 31 mg. of quinol were present or 0·3% of the potassium phenylsulphate fed.

We attempted to isolate the quinol as a *p*-toluenesulphonic ester, but without success. Our main product was phenol *p*-toluenesulphonate, m.p. 92° (12·7 g. or 54% of the dose).

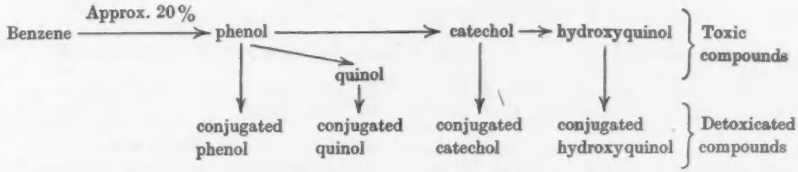
(2) *The glucuronide fraction of phenylsulphuric acid urine.* The above partially hydrolysed urine was warmed to expel ether and then made 10 N with respect to H_2SO_4. The mixture was refluxed for 1 hr. and subjected to continuous ether extraction. Only slight traces of phenol were found. This finding confirms the view that the administration of potassium phenylsulphate does not lead to an increased excretion of phenolic glucuronides.

IV. *The metabolites of phenylglucuronide*

Two rabbits were each given by stomach tube an aqueous solution containing 1·5 g. of phenylglucuronide dihydrate. Attempts to isolate the phenols of the ethereal sulphate and glucuronide fractions were not made in this case. We were, however, able to detect, besides phenol, definite but small amounts of catechol, quinol and hydroxyquinol in the ethereal sulphate fraction. In the glucuronide fraction the only hydroxybenzene present was phenol.

DISCUSSION

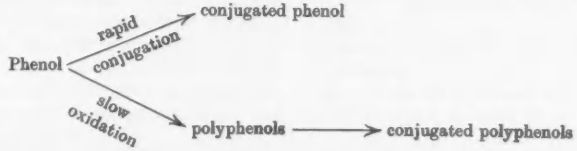
On the basis of the results of the present paper and of those of Porteous & Williams (1949*a*, *b*) and Garton & Williams (1948, 1949), the following scheme for the metabolism of benzene to hydroxyquinol is suggested.



This scheme accounts for only 20% of the benzene administered. Such aspects as the elimination of unchanged benzene (which may account for more than 50%), the formation of muconic acid and phenylmercapturic acid have yet to be considered and are being investigated by us.

According to the above scheme it is free phenol and not conjugated phenol which gives rise to polyphenols. The evidence for this view will occupy the rest of this discussion.

Porteous & Williams (1949*a*) have shown that about 21% of the benzene administered orally to rabbits is oxidized to phenols and the ratio phenol/polyphenols of the urine is roughly 1, i.e. phenol and the total polyphenols (catechol, quinol and hydroxyquinol) are produced in roughly equal amounts. On administration of phenol, however, the ratio phenol/polyphenols is about 5 or 6. This means that administered phenol is rapidly conjugated forming phenylsulphuric acid and phenylglucuronide which are (see p. 162) excreted unchanged. This rapid conjugation leaves only a small proportion of free phenol for further oxidation, unless there is a reversal of conjugation, thus:

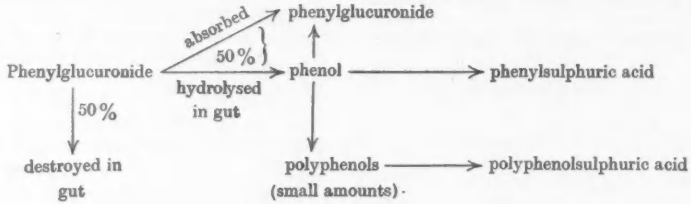


That the glucuronic acid and ethereal sulphate conjugations are rapid processes is now fairly well established (see Hemingway, Pryde & Williams, 1934; Sperber, 1948*a*).

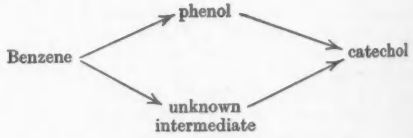
Earlier workers have shown that the potassium or sodium salts of phenylsulphuric acid whether fed by mouth or injected are rapidly excreted unchanged almost quantitatively (Christiani, 1878; Rhode, 1923; Sperber, 1948*b*). Our results confirm this view for we recovered 90 % of orally administered potassium phenylsulphate within 24 hr. of feeding. If this compound had undergone hydrolysis in the body to any significant extent, then a rise in the glucuronic acid output would have been noted. No such rise was observed even when the dose of the ethereal sulphate was as high as 500 mg./kg. and the conclusion was confirmed by our inability to find phenylglucuronide when the glucuronide fraction of the urine was examined qualitatively. We did find, however, in the ethereal sulphate fraction of 'potassium phenylsulphate urine' small amounts of quinol (estimated as 0·3 % of the dose) and extremely small traces of catechol and hydroxyquinol. These could have arisen from a small amount of free phenol released by a slight hydrolysis of phenylsulphuric acid by the phenolsulphatase of the gut. However, the possibility of direct oxidation of phenylsulphuric acid to quinolmonosulphuric acid cannot be completely ruled out, although we feel that this is unlikely because a strong acid such as phenylsulphuric acid, once in the body, would be too rapidly excreted to allow much oxidation. Both phenylsulphuric acid and phenylglucuronide are eliminated by renal tubular excretion in hens (Sperber, 1948*b*).

On injection, phenylglucuronide is excreted quantitatively unchanged in 24 hr. thus confirming the work of Nakano (1937) on rabbits and of Sperber (1948*b*) on hens. If any hydrolysis had taken place then a rise in ethereal sulphate would have occurred (see Fig. 1). It is clear that glucuronidase does not exert its hydrolytic function in the intact animal under the conditions of our experiments.

On oral administration, however, phenylglucuronide undergoes considerable hydrolysis and probably destruction. Only about 50 % of the dose could be accounted for in the urine, thus confirming the work of Nakano (1937). On the first day after feeding there is a sharp rise in ethereal sulphate corresponding to 15–20 % of the dose of glucuronide. Furthermore, in the ethereal sulphate fraction of the urine, phenol, quinol, catechol and hydroxyquinol are detectable. The excretion of glucuronic acid remains above normal for 3 days after dosing (see Fig. 2), and this probably means that the glucuronide is poorly absorbed from the intestine. Poor absorption of the glucuronide from the intestine would thus allow considerable hydrolysis and destruction by intestinal bacteria (Barber, Brooksbank & Haslewood (1948) have shown that certain bacteria destroy glucuronides). The fate of phenylglucuronide on oral administration can be represented as follows:



There is one further point which requires discussion and that is the conversion of phenol to catechol. The semi-quantitative results of Porteous & Williams (1949*a*, *b*) show that catechol and quinol are produced during the metabolism of benzene in equal amounts, whereas with phenol ten times as much quinol is produced as catechol. Quinol is thus the main oxidation product of phenol. This could mean that catechol is produced from benzene in more than one way, thus:



Such an intermediate could be 1:2-dihydro-1:2-dihydroxybenzene which could yield both phenol and catechol as suggested by Porteous & Williams (1949*b*).

SUMMARY

1. The metabolic fates of phenol, phenylsulphuric acid and phenylglucuronide have been studied in the rabbit.

2. Besides forming the main metabolites, phenylsulphuric acid and phenylglucuronide, phenol is oxidized to a small extent to quinol, catechol and hydroxyquinol, which occur only in the ethereal sulphate fraction of the urine. The main oxidation product is quinol. Both quinol and catechol were isolated as *p*-toluenesulphonates from phenol urine.

3. Orally administered potassium phenylsulphate and injected phenylglucuronide are excreted almost quantitatively unchanged by the rabbit.

4. Orally administered phenylglucuronide causes a considerable rise in ethereal sulphate output and small amounts of quinol, catechol and hydroxyquinol are also excreted. The evidence suggests that phenylglucuronide is partly hydrolysed in the gut yielding free phenol which is then oxidized to polyphenols.

5. The results are discussed and the conclusion is reached that the polyphenols of benzene and phenol metabolism are products of the oxidation of free phenol and not of conjugated phenol.

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The Biogenesis of Porphyrins

THE DISTRIBUTION OF ^{15}N IN THE RING SYSTEM*

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It was shown by Shemin & Rittenberg (1946*a*, *b*) that feeding of glycine labelled with ^{15}N to men or rats produces a relatively high isotope content in the porphyrin moiety of the haemoglobin molecule. The effect appeared to be specific, since ammonia, glutamic acid, leucine and proline, also labelled with the stable nitrogen isotope, were relatively ineffective in this respect. These experiments did not indicate whether the isotopic nitrogen was equally distributed over the four rings of protoporphyrin, and this paper describes in the first place experiments designed to investigate this question. Degradation of porphyrins which is required for this purpose can be done by either an oxidative or a reductive procedure. The latter gives rise to alkylated pyrroles which are fairly unstable to light and air. After some preliminary experiments, in which reduction with sodium alkoxides was used, this method was abandoned. The method finally chosen consisted of reduction of protoporphyrin to mesoporphyrin, followed by oxidation of the latter with chromic anhydride. The main products of this oxidation are methylethylmaleic acid imide and 1-methyl-2-2′-carboxyethylmaleic acid imide (haematinic acid imide).† The neutral compound is derived from the rings I and II and the acid is produced from the two acidic rings III and IV. The chromic anhydride oxidation has been used extensively in the porphyrin field and has been examined systematically by Fischer & Wenderoth (1939). However, their method could not be successfully applied to the small quantities of porphyrins available to us, and it was necessary therefore to study the conditions of oxidation of mesoporphyrin and the separation of the products in the pure state in some detail. An investigation of

* Preliminary communication, see Muir and Neuberger, 1948.

† In the German literature the term haematinic acid is used both for the above and for the corresponding diketodihydrofuran derivative; we propose to describe the former as haematinic acid imide and the latter as haematinic acid anhydride.

the ultraviolet absorption spectra, dissociation constants and solubilities in various solvents of the various maleic acid derivatives was therefore undertaken, and on this basis a method of oxidation and separation elaborated which permitted the isolation of the two imides in crystalline form on a scale of about 10–15 mg. A convenient synthesis of the two imides is also described in the Experimental section.

By using glycine labelled with ^{14}C on the carboxyl carbon Grinstein, Kamen & Moore (1948) showed that the carboxyl group of glycine does not appear in the protoporphyrin molecule. It thus appeared possible that the immediate precursor of part of the haemin molecule is not glycine, but another nitrogenous compound derived from it. One possible pathway appeared in the conversion of glycine to serine which has been shown to occur *in vitro* (Winnick, Friedberg & Greenberg, 1948) and the decarboxylation of the latter to ethanolamine. This hypothesis was tested by feeding ethanolamine, labelled with ^{15}N.

EXPERIMENTAL

Preparation of methylethylmaleic acid imide and of haematinic acid imide

Methylethylmaleic anhydride and haematinic acid anhydride have been prepared by addition of hydrogen cyanide in a heterogeneous medium to the corresponding substituted acetoacetates, followed by hydrolysis and dehydration (Michael & Tissot, 1892; Küster, 1906, 1908, 1924; Küster & Weller, 1917). We have found that the yields are improved and the preparation of the cyanohydrin simplified if the condensation of the acetoacetate is carried out with liquid hydrogen cyanide under the catalytic influence of a small amount of potassium cyanide (Linstead & Millidge, 1936). Küster (1908, 1924) prepared the imides from the anhydrides by treatment with ammonia; yields by this method are not good and separation from other products is rather laborious. It was found that the imides can conveniently be prepared by heating the anhydrides with urea (see Cohen, 1928). This method, which gives very good yields with disubstituted maleic acid anhydrides, cannot be used for maleic anhydride itself and gives only poor yields with citraconic anhydride.

Methylethylmaleic acid anhydride. Ethyl 1-ethylacetoacetate (174 g.; b.p. 97°/18 mm.) was added slowly at −5° to liquid HCN (51 g.) containing saturated aqueous KCN (1 ml.). The mixture was left at 0° for 18 hr. and then at 20° for 2 hr.; it was then poured into 36% (w/v) HCl (700 ml.) previously cooled to 0°. The solution was left for 3 days at 0°, and after removal of excess HCN by aeration was heated first to 50° for 3 hr. and then to 100° for 12 hr. On cooling NH_4Cl crystallized out and was removed by filtration. The solution was evaporated to dryness *in vacuo* and the residue extracted with dry boiling ethyl acetate (800 ml.). After drying and removal of the solvent, the residue was distilled at 210–220° (60 mm.). The mixture of anhydride and water was separated after addition of NaCl. The anhydride was redistilled first at atmospheric and then under reduced pressure; boiling point was 233–234°/760 mm. and 121–123°/18 mm. Yield was 55% of the theoretical.

Methylethylmaleic acid imide. The above anhydride (14 g.) and urea (6·5 g.) were heated together at 160° in a flask fitted with an air condenser until the evolution of NH_3 ceased (40 min.). The solid, including the copious sublimate, was taken up in a small amount of boiling water and allowed to cool. The crystalline material was filtered off and recrystallized from water; it had m.p. 68° which did not change on further recrystallization. (Found: N, 9·9; calc. for $C_7H_9O_2N$: N, 10·1%.) Yield was 70% of the theoretical.

Haematinic acid anhydride. Ethyl 1-acetylglutarate (185 g.; Clemo & Welch, 1928) was added slowly at −5° to liquid HCN (26 g.) containing 0·7 ml. of saturated aqueous KCN. The mixture was left at 0° for 18 hr. and at 20° for 2–3 hr. Hydrolysis of the cyanohydrin was carried out as described above. The solution, after being heated at 100° for 12 hr., was evaporated *in vacuo* to dryness and the residue extracted repeatedly with dry boiling ethyl acetate (1·5 l.). The combined extracts were dried and the solvent removed *in vacuo*. The residue was dissolved in water (600 ml.) and extracted thrice with $CHCl_3$. The aqueous solution was again evaporated to dryness and the residue taken up in ethyl acetate. The solvent was removed *in vacuo* until the volume of the solution was about 300 ml.; 50 ml. portions were now heated in a flask immersed in a bath which was slowly heated to 185°. A second thermometer was immersed in the liquid. Whilst the dehydration proceeded the inside temperature was considerably lower than the outside. At the end of the reaction this difference became small (5–10°). If the heating was prolonged beyond this point decarboxylation occurred. The solid which formed on cooling was dissolved in hot water (40 ml.) and extracted with $CHCl_3$ (300 ml.). The $CHCl_3$ extracts yielded after drying and evaporation *in vacuo* a crystalline residue which was recrystallized from ether and light petroleum (60–80°). Melting point was 99° (uncorr.). Yield was 65%.

Haematinic acid imide. A mixture of the haematinic acid anhydride (3·65 g.) and urea (1·5 g.) was heated at 150–155° for 40 min. The residue was dissolved in water (10 ml.) and 36% (w/v) HCl (4 ml.) was added. The solution was extracted with ethyl acetate. The crystalline material which was left after removal of the solvent was recrystallized from ether and light petroleum (b.p. 60–80°). The melting point was 110° and the yield 75%. On recrystallizing the material twice from a little water the melting point rose to 117° (uncorr.). (Found: N, 7·7; calc. for $C_8H_9O_4N$: N, 7·6%.)

The methyl ester of the imide was obtained as follows: 0·5 g. of the imide acid was dissolved in cold anhydrous methanol (20 ml.) containing HCl (1 N). The solution was left at 0° for 40 hr. and then concentrated *in vacuo*. The residue was dissolved in ether (40 ml.) and extracted twice with $NaHCO_3$ solution and once with saturated NaCl (3 ml.). The ether solution was dried and the solvent removed. The residue was distilled at 5 mm. pressure at 160°. The distillate, which soon crystallized, was dissolved in $CHCl_3$ and precipitated by light petroleum (b.p. 40–60°). Melting point was 65° (uncorr.); Küster (1908) gives m.p. 64°. (Found: N, 7·0; calc. for $C_9H_{11}O_4N$: N, 7·1%.)

Properties of the maleic acid derivatives

Ultraviolet absorption spectra. A Beckman photoelectric spectrophotometer was used. The results are shown in Figs. 1, 2 and 3. Both imides have a maximum in water, which is not very sharp, at 293–5 mμ.; this is shifted in ether to about 275 mμ. Methylethylmaleic anhydride is very insoluble in water, and its absorption was therefore measured in ether. The two anhydrides have no maximum above 260 mμ., and their absorption at 310 mμ. is very small, i.e. only about 10% of that of the corresponding imides. Measurement of ultraviolet absorption at 275, 295 and 310 mμ. can thus be

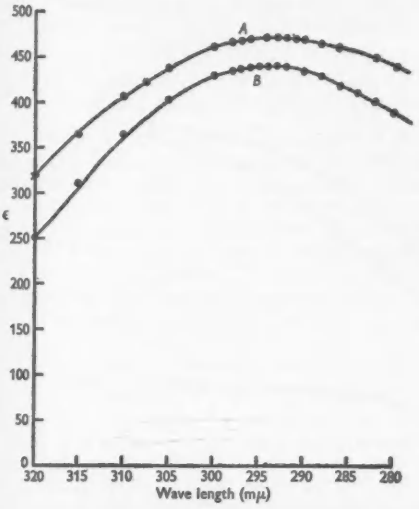

Fig. 1. Absorption spectrum of haematinic acid imide (*A*) and methylethylmaleic acid imide (*B*) in water. ε in Figs. 1, 2 and 3 denotes molecular extinction coefficient.

used to determine approximately the relative concentrations of imides and anhydrides in a mixture, provided that other substances absorbing in this region are absent. The relatively intense absorption of the four disubstituted maleic acid derivatives in the region 250–300 mμ. is in marked contrast to the weak absorption of maleic anhydride itself (Friedl, 1924).

The ultraviolet absorptions of the two haematinic acids in ether have been measured by Friedl (1924).

Dissociation constants. The apparent dissociation constants of the two haematinic acids were determined by titrating 20 ml. portions of 0·01 N-solutions with 0·1 N-sodium hydroxide at 22 ± 1° using a Muirhead pH meter calibrated in the usual manner. The results are shown in Fig. 4. Haematinic acid

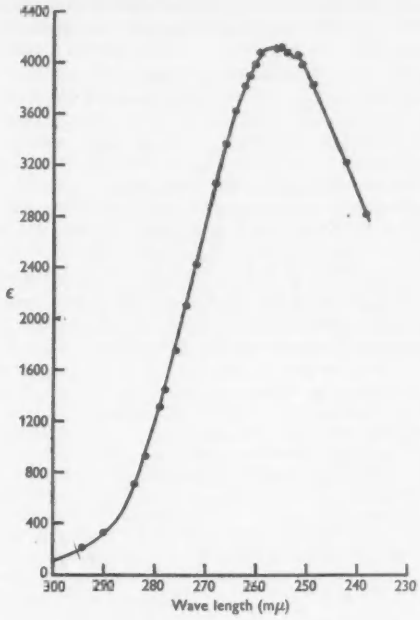

Fig. 2. Absorption spectrum of methylethylmaleic acid anhydride in ether.

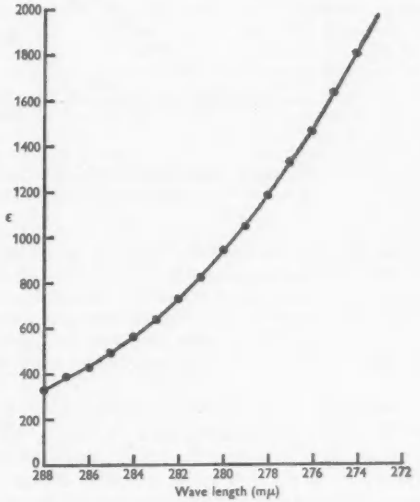

Fig. 3. Absorption spectrum of haematinic acid anhydride in water.

imide behaves as a monobasic acid with an apparent pK of 4·53. No correction for ionic strength has been made and the value is of the expected order of magnitude. Haematinic acid anhydride behaves as a tribasic acid, but even an approximate calculation of the first and the second pK is not easy. Haematinic acid anhydride may exist in water wholly or partly as acid anhydride which opens instantaneously on addition of alkali. The equilibrium constant for this reaction cannot be calculated from our data. It may suffice therefore to give the mid-points of three steps in the titration; these are as follows: 3·75; 5·08; 6·46. It is clear that haematinic acid anhydride is a much stronger acid than the imide and it appeared possible to base a method of separation of the two compounds on this difference.

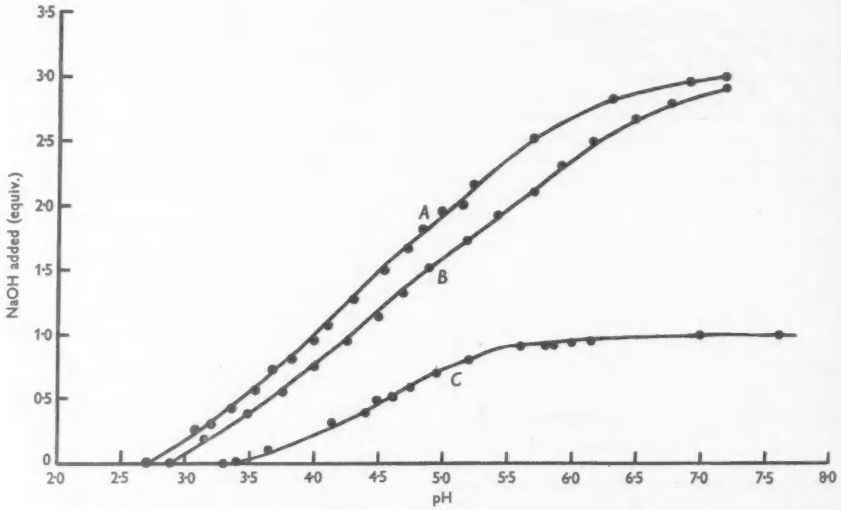

Fig. 4. Electrometric titration of haematinic acid anhydride with (*A*) and without (*B*) calcium chloride, and of haematinic acid imide (*C*).

Fig. 4 also shows that addition of calcium chloride causes an apparent increase in the pK values of haematinic acid anhydride. This effect, due to complex formation, is observed with some other di- and tri-carboxylic acids and can be utilized for the separation of the anhydride from the imide.

Solubilities. Approximate distribution coefficients were determined by shaking aqueous 0·001–0·002 M-solutions of the two haematinic acids and of methylethylmaleic acid imide with an equal volume of organic solvent previously saturated with water. Distribution coefficients were obtained from the difference of ultraviolet absorption of the aqueous phase, before and after equilibration with the organic solvent. Methylethylmaleic anhydride is insoluble in water and easily soluble in all organic solvents tried. Its removal from an oxidation mixture thus presents no difficulty. The solubility of the two haematinic acids in ether is about the same as in water, and this solvent, which was used by Fischer & Wenderoth (1939), is therefore not convenient for the complete extraction of the imides. This can be done more easily with ethyl acetate, for which distribution coefficients of 15–20 were observed. Haematinic acid anhydride and methylethylmaleic acid imide are much more soluble in chloroform than in water, whilst the reverse was found for haematinic acid imide, the distribution coefficient for the latter being about 0·033.

Separation of haematinic acid imide and anhydride

At pH 5·45 about 90% of the imide (pK 4·5) should exist as salt, and 10% as the free acid. However, extraction with ethyl acetate, in which the imide is about 15 times more soluble than in water, should remove more than half of the imide, if the pH is kept constant with a buffer which is not extracted by the organic solvent. The anhydride, on the other hand, should exist at that pH as a mixture of dibasic and monobasic salts, and thus not be extracted to any extent by ethyl acetate. This prediction was put to the test: 9·2 mg. of each acid were dissolved in 50 ml. 0·5 M-citrate, pH 5·44, and extracted with ethyl acetate. Spectrophotometric examination of the two aqueous phases showed that about

44% of the imide and almost none of the anhydride was extracted under these conditions. That a smaller amount of imide was extracted than was anticipated is probably due to a decrease in apparent pK of haematinic acid imide in the highly concentrated citrate buffer used. This method was also found to separate oxalic acid and succinic acid, which have been identified as by-products of porphyrin oxidation, from haematinic acid imide.

Oxidation of mesoporphyrin methyl ester by chromic anhydride

Under the usual conditions (Fischer & Wenderoth, 1939) the ester is first hydrolysed by 50% (v/v) sulphuric acid and then oxidized by chromic anhydride, after addition of an equal volume of water. The formation of methylethylmaleic acid imide and haematinic acid imide is expressed by the following equation:

$$3C_{36}H_{42}O_4N_4 + 46CrO_3 + 69H_2SO_4 = 6C_7H_9O_2N + 6C_8H_9O_4N + 18CO_2 + 23Cr_2(SO_4)_3 + 78H_2O. \quad (1)$$

According to this equation twenty-three O atoms are consumed, six for the oxidation of the methanol and seventeen for that of the porphyrin. Of the six molecules of carbon dioxide produced, two are derived from methanol and four from the methyne groups. In view of the importance of this oxidation for tracer studies generally, it was desirable to establish whether this equation represents the main or only pathway of oxidation.

Stability of maleic acid imides to chromic anhydride and sulphuric acid

Samples (56 mg.) of methylethylmaleic acid imide were dissolved in 50 ml. of water containing varying concentrations of H_2SO_4 (12·5, 25, 50, % all v/v), and CrO_3 (160 mg.). The solutions were kept at 0, 20 and 37° respectively, and 5 ml. samples were withdrawn and CrO_3 was titrated iodometrically. There was no change in the iodine titration, apart from one sample which had been left for 24 hr. at 37°. In the latter, the figures indicated that about 4% of the CrO_3 had been consumed. Similar results were obtained with haematinic acid imide. The possibility that the acid might hydrolyse the imides to anhydrides was also investigated. Haematinic acid imide (10·2 mg.) was dissolved in 50% (v/v) H_2SO_4 (10 ml.); 1 ml. was diluted at once with water to 10 ml. and the ultraviolet absorption measured from 285 to 310 mμ. After standing for 3·5 hr. at 23°, 1 ml. was again withdrawn, diluted to 10 ml. and its extinction curve measured. There was no change, and the values agreed closely with those expected for the imide. Another series of experiments was carried out as follows: haematinic acid imide (150 mg.) was dissolved in water (35 ml.); to 5 ml. portions were added, 1, 2, 3, 4 and 5 ml. respectively of a 50% (v/v) H_2SO_4, and the volume was made up with water to 10 ml. These solutions were allowed to stand either at 0° or at 23° for 4 hr., and were then extracted 3 times with ethyl acetate (20 ml. each), which removed the unchanged imide. Estimation of NH_3 in the aqueous fractions in a Kjeldahl apparatus showed that no NH_3 had been formed. It is concluded, therefore, that the two imides are stable, both to the oxidative action of CrO_3 and to the hydrolytic effect of H_2SO_4 under the conditions used for the oxidation of porphyrins.

Oxidation of mesoporphyrin. This was investigated by following the consumption of chromic anhydride, the production of carbon dioxide, and the formation of the two imides and other possible compounds.

In a typical experiment, mesoporphyrin methyl ester (250 mg.) was dissolved in 50% (v/v) H_2SO_4 (15 ml.) and the solution shaken for 2 hr. at 18–20°. The solution, now containing free mesoporphyrin, was cooled to 0°, and CrO_3 (725 mg.; 10% excess) dissolved in 15 ml. water previously cooled to 0°, was added. The mixture was then shaken at about 18°, whilst N_2, freed of CO_2, was passed through. CO_2 evolved was collected in three flasks containing measured amounts of 0·1 N-$Ba(OH)_2$. After suitable intervals the $Ba(OH)_2$ solutions were removed, acetone (0·8 vol.) added and the $Ba(OH)_2$ titrated with 0·1 N-HCl, using thymol blue as internal indicator. After 5 hr. the mixture was extracted three times with 30 ml. portions of ethyl acetate, the combined extracts were washed with saturated NaCl (5 ml.) and the salt solution again extracted with ethyl acetate (10 ml.). The combined ethyl acetate solutions were dried and the solvent removed *in vacuo*. The weight of the residue was 201·4 mg. The yield at this stage would be 75%, if the residue consisted entirely of the two imides. The aqueous fractions were used for estimation of total N and NH_3-N. The residue from the ethyl acetate extractions was dissolved in boiling water (10 ml.) and filtered. After cooling to 0°, the pH was brought to 7·0 by addition of $NaHCO_3$ (100 mg.). The solution was now extracted with $CHCl_3$ (3 × 30 ml.) and the combined $CHCl_3$ solutions were washed with saturated NaCl and dried. On removing the solvent, 71·6 mg. of almost pure methylethylmaleic acid imide were obtained. This was dissolved in a little hot water and crystallized by slow evaporation in a partially evacuated desiccator. There were obtained 58·5 mg. of the imide of m.p. 68°. (Found: N (Kjeldahl) 10·2; calc. for $C_7H_9O_2N$: N, 10·1%.) Yield of crude imide was 63% of the theoretical. The neutral aqueous solution was acidified with N-HCl (0·5 ml.) to pH 5 and 20 ml. of 0·5 M-citrate buffer, pH 5·44, were added. The solution was extracted with ethyl acetate (4 × 65 ml.). The combined ethyl acetate solutions were extracted twice with 10 ml. portions of N-HCl containing 10% (w/v) NaCl and once with saturated NaCl (5 ml.). The combined aqueous solutions were again extracted with ethyl acetate (10 ml.). From the combined ethyl acetate solutions there were obtained 116·7 mg. of a partly crystalline solid. Ultraviolet absorption of an aqueous solution of this material revealed the presence of some material absorbing at about 300 mμ. other than haematinic acid imide or anhydride. The solid was therefore dissolved in water (10 ml.), extracted twice with $CHCl_3$ (1 ml.) to remove impurities and then with ethyl acetate (3 × 20 ml.). On concentrating the dried ethyl acetate solutions, crystals were obtained which were recrystallized from a very small amount of water, giving 80·5 mg. of imide of correct melting point, analysis and ultraviolet absorption spectrum. In a few experiments the material was still not quite pure and it was then sublimed at 0·001 mm. pressure at a bath temperature of 150–155°. The oxidation and separation were carried out with 50 mg. of mesoporphyrin methyl ester and the yields of the two imides were only slightly lower. In several experi-

ments it was desired to measure the CrO_3 consumption; this was done by removing samples from the oxidation mixture, at intervals, and titrating the CrO_3 iodometrically.

The results of the various estimations may be described as follows: at 0° oxidation was very slow, partly at least owing to the insolubility of the porphyrin. The oxidation appeared to be complete at 18–20° within 5 hr.; after that time there was no further evolution of carbon dioxide or uptake of oxygen. The mesoporphyrin disappeared during the first 2 hr., as shown by spectroscopic examination. However, figures for carbon dioxide evolution or chromic anhydride consumption never reached the values required by equation (1). In several experiments it was found that between 68 and 75 % of the theoretical amount of carbon dioxide was produced in the first 1·5 hr., and after 5 hr. the values were from 82 to 86 %. The oxygen uptake showed a similar course, about 85 % being observed after 5 hr. The oxidation of methanol was examined by following carbon dioxide evolution and chromic anhydride consumption, and was found to be similar to that of mesoporphyrin ester. The 5 hr. values were slightly higher and varied between 85 and 94 % of the theoretical. The two imides could never be obtained in a yield higher than 66 %, and the mechanical losses as judged from recovery experiments are not likely to exceed 5 %. Moreover, it was found that 12–16 % of the total nitrogen was present in the first aqueous fraction, not extractable by ethyl acetate. This nitrogen appeared as ammonia on mild treatment with alkali and must therefore be present either as ammonia or as a very labile amide. This observation, and the reported presence of volatile acids, maleic acid anhydrides and of oxalic and succinic acids in the oxidation products of porphyrins (Küster, 1922; Fischer & Wenderoth, 1939), leads to the conclusion that either the intermediate in the oxidation is more sensitive to oxidative or hydrolytic attack than the imides, or that there is a side reaction not leading through the maleic acid imides. This oxidative reaction cannot account for more than 15 % of the mesoporphyrins, as judged from the amount of ammonia produced.

However, if this more complete oxidation were the only side reaction, it would be expected that the values for oxygen consumption and carbon dioxide evolution should be higher than those predicted by equation (1). In fact, the figures were lower by 14–18 % than those calculated. Since the mesoporphyrin has disappeared at the end of the oxidation it would follow that another reaction must occur, giving products, possibly dipyrryl derivatives, which are relatively resistant to further oxidation by chromic anhydride. This would also explain the presence of material giving an intense absorption at 300 mμ. in the haematinic acid fraction. It was also observed that the nitrogen content of this fraction was much higher than could be accounted for by the haematinic acid imide. It can thus be concluded that 63–70 % of the oxidation products of mesoporphyrin consist of methylethylmaleic acid imide and haematinic acid imide, the rest being accounted for by more complex substances of unknown structure; and secondly, by more completely oxidized substances arising from a side reaction.

These results have an important bearing on degradation experiments in tracer studies of porphyrins, both in this investigation and in other studies under way. The porphyrin structure contains two pyrrolenine, one pyrrol and one maleic acid imide nucleus. The three structures may be expected to give rise on oxidation with chromic anhydride to varying amounts of maleic acid imide derivatives, and if bond fixation were to occur (cf. Fischer & Orth, 1937) it would not be permissible to assume that methylethylmaleic acid imide was derived to an equal extent from rings I and II, and haematinic acid imide from rings III and IV. In neutral solution tautomerism is possible since only two of the four nitrogen atoms carry hydrogens. However, oxidation takes place in strongly acid solution, in which the mesoporphyrin is presumably present as bivalent cation. In such a structure tautomerism is impossible (see Corwin, 1943) and we may suppose that complete resonance occurs. We assume that all three structures make similar contributions to the four nuclei, and that the two imides are derived to an equal extent from rings I and II or III or IV respectively. In any case, the fact that the two imides could be isolated in yields well above 50 % indicates clearly that each of the two compounds cannot be derived from one nucleus only.

Another, less theoretical, point concerns the source of the carbon dioxide evolved during the oxidation. The major part must arise, as required by equation (1), from the four methyne and the two methanol carbon atoms. However, the side reaction which produces ammonia and volatile acids will presumably yield some carbon dioxide from the pyrrolic nuclei in addition to that derived from methyne carbon atoms. From the amount of ammonia present in the aqueous phase it can be estimated that this reaction cannot account for more than 15 % of the mesoporphyrin oxidized. From this calculation it follows that about 10 % of the carbon dioxide evolved may arise from carbon atoms other than the methyne and methanol carbon atoms. On this basis approximately 60 % of the carbon dioxide evolved may be ascribed to the methyne carbon atoms, 30 % to the methanol, and 10 % to unknown sources.

Biological experiments

Preparation of isotopic compounds. Glycine containing 31·69 atom % excess ^{15}N was prepared from phthalimide and ethyl chloroacetate (Schoenheimer & Ratner, 1939), and

ethanolamine hydrochloride with 31·65 atom % excess ^{15}N, as described by Bloch & Schoenheimer (1941).

Administration of compounds and care of animals. Male and female Hollingsworth half-lopped rabbits (1·5–1·8 kg. body weight), and rats of the black-and-white Institute strains, (280–320 g. body weight) were used. The rabbits were fed the ordinary stock diet, whilst the rats, from 5 days before the administration of the isotopic compounds, were given a semi-synthetic diet with a protein content of 5%. Two days after the feeding of the labelled substances had finished, the rats were put back on the stock diet containing 18% protein. The animals were kept in metabolism cages of the usual design. The isotopic compounds were fed on 5 successive days in equal doses to animals which had been fasting for about 18 hr. The glycine and ethanolamine hydrochloride were dissolved in water to give a 5% solution which was used to moisten a weighed quantity of the dry, pelleted food, representing about 40% of the normal daily ration. The animals usually consumed the food within 2–3 hr. and any scattered pellets were collected and weighed. The correction for this loss was negligible in almost all experiments. Each of 5 rabbits consumed altogether 0·6 mmol. glycine/100 g. body weight. Four rats were each given 0·8 mmol. ethanolamine/rat, whilst three rats received glycine, the dose amounting to 0·6 mmol./rat. The animals were killed on the fifteenth or seventeenth day after the last administration of the isotopic compound.

Isolation of mesoporphyrin. The rabbits were anaesthetized with ether, and blood collected from the aorta. In several experiments the animals were perfused through the vena cava with heparin-containing saline. The heparinized blood was centrifuged, the red cells were twice washed with saline, and then lysed by addition of an equal volume of water containing saponin. In the earlier experiments haemin was isolated by adding the haemoglobin solution slowly, at 95°, to 5 vol. glacial acetic acid containing 1% (v/v) saturated NaCl solution. The solution was allowed to cool very slowly to room temperature and was then left at 0° for 18 hr. The haemin crystals were separated and washed with acetic acid, water, ethanol and ether. Haemin was then dissolved in methanol containing 10% (w/v) anhydrous oxalic acid, about 10 ml. being used for 50 mg. haemin. The further procedure followed that of Grinstein (1947). The protoporphyrin methyl ester was recrystallized, if the melting point was lower than 220°. In later experiments the method of Grinstein was used from the beginning.

The reduction of protoporphyrin to mesoporphyrin ester was done either by the HI method of Grinstein & Watson (1943), or by a catalytic method similar to that of Granick (1948), who used colloidal Pd. In agreement with other workers, it was observed that, with the usual preparations of Pd or Pt catalysts, no reduction to mesoporphyrin took place. However, a catalyst of the polyvinyl type described by Rampino & Nord (1941) was found to be effective. Reduction by the latter method, which gave slightly higher yields than the HI method, was done as follows: 0·275 g. of polymerized methyl methacrylate (average mol. wt. 1×10^6) was dissolved in anhydrous formic acid. The latter had been dried over $CuSO_4$, distilled *in vacuo*, and crystallized at 0°. $PdCl_2$ solution (0·25 ml.), containing 15 mg. Pd, was then added. H_2 was passed in for 3–4 min. and $NaHCO_3$ (0·045 g.) in water (0·25 ml.) added. As soon as all the Pd had been reduced (about 1 min.) protoporphyrin methyl ester (0·15–0·2 g.) was added. The solution was kept at 52–55°, while a slow stream of H_2 was passed through. When reduction was complete, as judged spectroscopically, the solution was poured into ether. The methyl methacrylate, which precipitated, was separated by decantation and repeatedly extracted with ether. The combined ether solutions were extracted with N-HCl and the latter with $CHCl_3$. The $CHCl_3$ solution was washed with water, dried, and concentrated *in vacuo*. The mesoporphyrin ester, which was crystallized from $CHCl_3$-methanol, was usually obtained in a yield of 70%, and had m.p. 212–214°. The oxidation to the maleic acid derivatives was carried out as described above.

In the experiments with rats no degradation was carried out; haemin was isolated as described above, and recrystallized by dissolving in pyridine-$CHCl_3$ and precipitating with hot glacial acetic acid containing NaCl. The isotope ratio was estimated with the mass spectrometer.

RESULTS AND DISCUSSION

Table 1 shows that the ^{15}N contents of the methylethylmaleic acid imide and of the haematinic acid imide fractions are almost identical in all six experiments. With only one rabbit (no. 1) is the difference

Table 1. ^{15}N *contents of methylethylmaleic acid imide and of haematinic acid imide obtained from the haemoglobin of rabbits fed isotopic glycine.*

(Glycine contained 31·69 atom % excess ^{15}N.)

		Atom % excess ^{15}N		
Rabbit no.	Sex	In acidic fraction	In non-acidic fraction	Difference
1	M.	0·108	0·120	0·012
2	F.	0·186	0·190	0·004
3	M.	0·086	0·092	0·006
4	M.	0·122	0·117	0·006
5	F.	0·156	0·150	0·006
6	M.	0·142	0·139	0·003

outside the limits of experimental error, but it is doubtful whether this isolated observation is significant. We may, therefore, conclude that the labelled nitrogen enters at a similar rate into the acidic and non-acidic nuclei of the haemin group. Haemoglobin contains four haemin groups and each haemin has four pyrrolic nuclei. However, the experiments reported here do not conclusively prove that all sixteen pyrrolic nuclei in a haemoglobin molecule contain similar amounts of ^{15}N. In the Experimental section of this paper arguments were adduced to show that the two imides are derived to an equal extent from rings I and II, and III and IV, respectively. Even if this is accepted, however, it is still possible that, say, rings I and III contain all the excess ^{15}N, whilst rings II and IV contain none. This possibility, although it may appear remote, cannot be excluded on the basis of the data reported here. However, unpublished results show that, taking into account the rate of formation of haemoglobin and the ^{15}N content of the glycine pool, the isotope

content in haemin is the highest theoretically possible. We may, therefore, accept the conclusion that all four pyrrolic nuclei of any one haemin group and all four haemin groups of the haemoglobin molecule have identical or closely similar isotope ratios. From this it follows that the four rings either arise from a common pyrrolic precursor, which is modified afterwards, or that the four rings are synthesized independently at similar or identical rates from glycine or a substance derived exclusively from glycine.

The experiments with rats are shown in Table 2. The ^{15}N contents of the haemin after feeding a rather small amount of isotopic glycine are of the expected order of magnitude. With ethanolamine, which was fed at a slightly higher molar concentration, only a small amount of incorporation of ^{15}N is observed. It is clear that ethanolamine cannot be an intermediate between glycine and protoporphyrin. The small amount of ^{15}N present in these experiments may be due to the fact that ethanolamine can be converted, probably largely through choline and betaine, to glycine (Stetten, 1941).

Table 2. ^{15}N *contents of haemin of rats which had received isotopic glycine or ethanolamine*

(Glycine and ethanolamine each had 31·69 atom % excess ^{15}N.)

Rat no.	Sex	Substance fed	Atom % excess ^{15}N of haemin
1	M.	Ethanolamine	0·006
2	F.	Ethanolamine	0·022
3	M.	Ethanolamine	0·016
4	F.	Ethanolamine	0·018
5	M.	Glycine	0·102
6	F.	Glycine	0·105

SUMMARY

1. A synthesis of methylethylmaleic acid imide and of haematinic acid imide, two oxidation products of mesoporphyrin, is described. The ultraviolet absorption spectra in water and the dissociation constants of these and related compounds have been determined.

2. The oxidation of mesoporphyrin by chromic anhydride was investigated, and conditions were established which permit the isolation of the two maleic acid imides on a micro-scale. This method was applied to the degradation of mesoporphyrin obtained from rabbits fed glycine containing an excess of ^{15}N. The results show that the acidic and non-acidic pyrrole nuclei in haemin have the same or closely similar isotope ratios.

3. Ethanolamine containing an excess of ^{15}N was fed to rats. The haemin obtained had a much lower ^{15}N content than that of control animals which had received a slightly smaller amount of labelled glycine. It is concluded that the nitrogen of ethanolamine is not used to any marked extent in the synthesis of protoporphyrin.

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Factors Affecting Synthesis of Ascorbic Acid in Cress Seedlings

2. ASCORBIC ACID SYNTHESIS IN RELATION TO SUGAR FORMATION

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In a previous paper (Mapson & Cruickshank, 1947) it was shown that certain salts stimulated the synthesis of ascorbic acid in cress seedlings and increased the pH of the cell sap, whereas other salts had the reverse action. It was concluded from this that the deciding factor was the amount of base made available to the plant after metabolism of the salt.

Ray (1934) found that certain sugars increased the production of ascorbic acid when supplied to the excised pea embryo. He calculated his results on the basis of concentration, but, as growth was affected by the various treatments, we have recalculated them as the amount produced by a given number of seedlings. Of all the substances tested by him, hexose sugars alone produced a significant increase in ascorbic acid. Of these fructose was the most effective, followed in descending order by mannose, glucose and galactose. He suggested, therefore, that the hexose sugars served as precursors of ascorbic acid.

In view of Ray's (1934) results it seemed desirable to ascertain whether the treatments described in our earlier paper (Mapson & Cruickshank, 1947) had affected the synthesis of ascorbic acid by reason of their effect on the formation of hexose sugars. We therefore followed the formation of hexoses and ascorbic acid in seedlings germinated in the presence of various salts. The salts chosen fell into three groups, viz. those which had been shown (*a*) to decrease the synthesis, (*b*) to increase the synthesis, and (*c*) to have little effect.

METHODS

Conditions of germination

The seeds were germinated under the conditions described in the earlier paper (Mapson & Cruickshank, 1947). In the present experiments, when sugar and a nitrogenous salt were added, it was found essential to add $HgCl_2$ (1/20,000) to the medium to prevent the growth of moulds. The Hg salt was also added to the controls. The seedlings were grown in the dark, to eliminate changes due to light or photosynthetic activity.

As in our previous communication, all results are expressed in terms of the amounts of the individual constituents produced by 100 seedlings, this having been found to be a more satisfactory basis for comparison than concentration.

Estimation of sugars

When sugars were added to the medium in which the seeds were grown, all samples of seedlings taken for sugar analysis were washed in several changes of distilled water to remove any adhering sugar. Sugars were extracted from the seedling with boiling 80% ethanol, the ethanol removed under reduced pressure and the residual liquid cleared by treatment with neutral Pb acetate; excess of Pb was removed by oxalate.

Total reducing sugars. The total reducing sugars were estimated in accordance with the method of Somogyi (1937) as modified by Hanes and described by Hockenhull & Herbert (1945). The reducing power has been expressed in equivalents of glucose.

Non-fermentable sugars. The non-fermentable reducing sugars and other non-fermentable reducing substances were estimated after removal of the fermentable sugars by yeast, as follows: 10 g. of baker's yeast ground with 100 ml. of water were allowed to stand 1 hr. at room temperature. The solution was centrifuged and the residue washed with a further 100 ml. of water and recentrifuged. The washed yeast was taken up in 100 ml. of phosphate buffer (0·05 M, pH 6·0). To 10 ml. of the sugar solution, 5 ml. of the yeast suspension were added. The combined solutions were incubated for 3 hr. at 37°, cooled and filtered through a fine paper and a measured volume taken for estimation of reducing titre.

Hexose sugars. Partition chromatography showed that the only hexose sugars present were glucose and fructose (Jermyn & Isherwood, 1949). These were calculated as being equivalent to the total reducing sugars minus the non-fermentable fraction (T.R.S. – N.F.).

Sucrose. Sucrose was estimated by determining the increase in reducing titre after hydrolysis by invertase. In the following Figures and Tables the value for sucrose is expressed in terms of its glucose equivalent.

Fructose. Fructose was estimated directly by the resorcinol method proposed by Roe (1934) and modified by Cole (1942, unpublished).

'*Glucose*'. The glucose content has been calculated as being equivalent to the hexose sugars minus fructose.

Ascorbic acid

Ascorbic acid was estimated by titration against 2:6-dichlorophenolindophenol, as in our previous investigations (Mapson & Cruickshank, 1947).

Fat (ether extract)

The seedlings were thoroughly ground up with quartz, dried with Na_2SO_4 and extracted with ethyl ether for 6 hr. The ether was evaporated and the residue taken up in light petroleum. Na_2SO_4 was again added to eliminate traces of water, the solution was filtered, and the light petroleum removed under reduced pressure. Acid values and iodine values were determined on the ether extract, the latter by Wijs's method.

pH of cell sap

The same technique was used as described by Mapson & Cruickshank (1947).

RESULTS

The food reserves of the cress seed

The cress (*Lepidum sativum*) belongs to the Cruciferae. Its seeds contain about 26 % of fat as their main food reserve. A small amount of sucrose is also present, but there is no starch or hexose sugar. Expressed on the basis of 100 seeds, the fat and carbohydrate reserves were as follows:

	(mg.)
Fat	55–60
Sucrose	10
Non-fermentable sugars, etc.	6·5

Changes in seeds germinated in water

When cress seeds were germinated in the dark in distilled water, the following changes were observed (Fig. 1). There was a rapid fall in sucrose, which

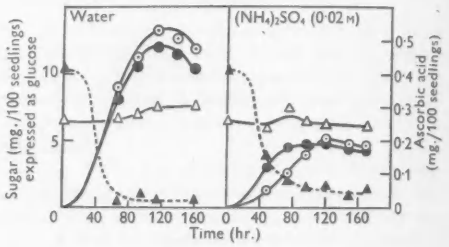


Fig. 1. Ascorbic acid and sugar content of seedlings germinated in water and $(NH_4)_2SO_4$; ▲—▲, sucrose; ⊙—⊙, hexose (T.R.S. – N.F.); △—△, non-fermentable sugars; ●—●, ascorbic acid.

reached a constant value at approximately 48 hr. During this period an appreciable amount of ascorbic acid was synthesized, but little or no fat had been mobilized (see Fig. 9). The non-fermentable sugars

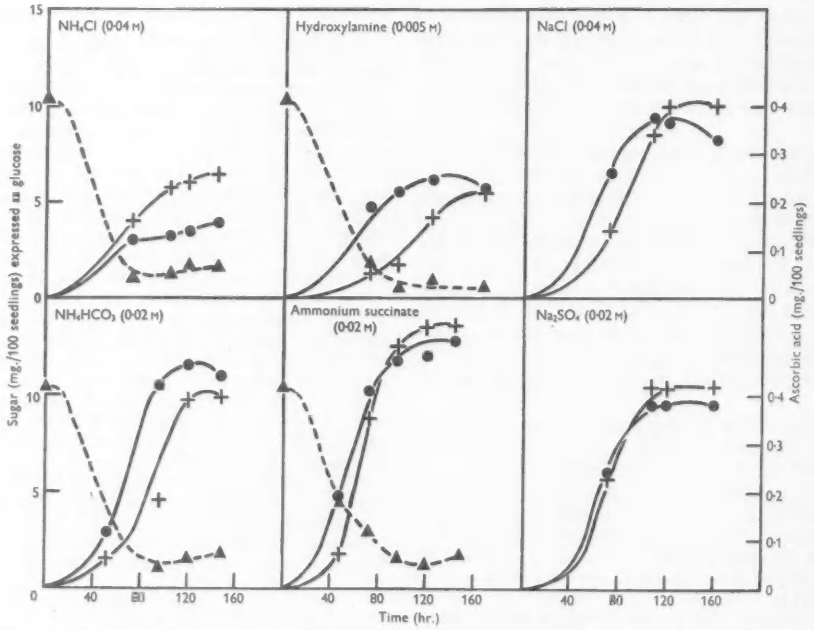


Fig. 2. Ascorbic acid and sugar content of cress seedlings germinated in ammonium and other salt solutions; ●—●, ascorbic acid; +—+, hexose (T.R.S. – N.F.); ▲—▲, sucrose.

remained at 6·5–7 mg./100 seedlings throughout the experimental period. The greatest change was in the hexoses, which reached a maximum at approximately 120 hr. of germination. The most significant observation was the close correspondence between the rise in hexose sugars and the rise in ascorbic acid.

maximum amount of hexose sugar and of ascorbic acid formed was, respectively, only 36 and 39 % of that formed in seedlings grown in water.

Similar experiments were carried out with NH_4Cl and $NH_2OH.HCl$ (Fig. 2), both of which decrease the formation of ascorbic acid and hexose sugars.

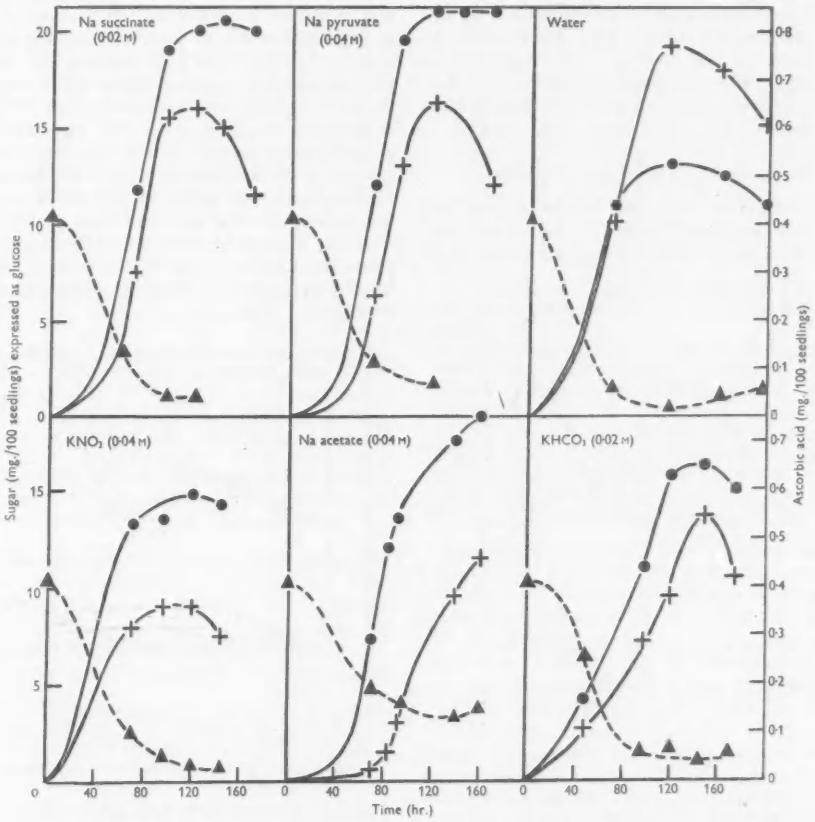


Fig. 3. Ascorbic acid and sugar content of cress germinated in sodium and potassium salts; ●—●, ascorbic acid; +—+, hexose (T.R.S. – N.F.); ▲—▲, sucrose.

Changes in seeds germinated in different ammonium salts

A similar experiment was carried out with seeds germinated in $(NH_4)_2SO_4$ (Fig. 1). Here, also, sucrose rapidly disappeared. The fall was slightly less rapid than in the seedlings grown in water, but this difference has not invariably been observed. The non-fermentable sugars again showed no change. The chief difference was the much smaller amount of hexoses formed, and this was associated with a correspondingly reduced synthesis of ascorbic acid. The

The changes found with NH_4Cl were essentially similar to those observed with $(NH_4)_2SO_4$, but with $NH_2OH.HCl$ the correspondence between the rates of production of these constituents was less close.

Our original experiments had shown that with ammonium salts such as the bicarbonate and succinate where both ions may be utilized by the plant, the synthesis of ascorbic acid is similar to that in seedlings grown in distilled water, but greater than in seedlings grown in $(NH_4)_2SO_4$ or NH_4Cl. This increased synthesis of ascorbic acid is associated with an increased formation of hexoses (Fig. 2).

Changes in seeds germinated in different sodium and potassium salts

When combined with a 'utilizable' anion, Na and K salts stimulate the synthesis of ascorbic acid; when the anion is non-utilizable, ascorbic acid is decreased (Mapson & Cruickshank, 1947). Reference to Figs. 2 and 3 again shows that with various Na and K salts, the formation of hexoses is closely related to that of ascorbic acid. Non-utilizable ions such as Cl^- and SO_4^{--}, when combined with ammonium, had a more depressing effect on both constituents than when combined with Na or K (cf. Figs. 1 and 3).

Changes induced by supplying glucose

This parallelism between hexose sugars and ascorbic acid supports Ray's (1934) hypothesis that ascorbic acid may be derived from such sugars. It

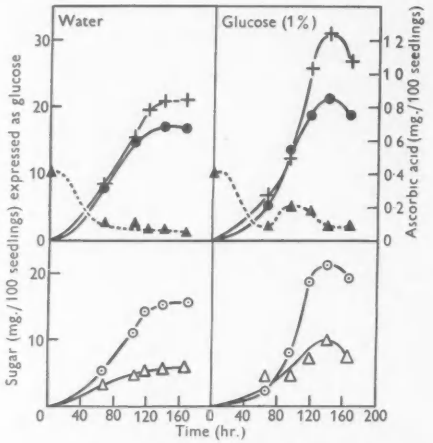


Fig. 4. Ascorbic acid and sugar contents of seeds germinated in water and glucose (1%) solution; ▲—▲, sucrose; +—+, hexose (T.R.S. – N.F.); ●—●, ascorbic acid; ⊙—⊙, glucose; △—△, fructose.

was therefore to be expected that increasing the latter in the seedling would increase the synthesis of ascorbic acid. To test this view cress seedlings were germinated in water and in a solution of 1% (w/v) of glucose (Fig. 4). During the first 90 hr. the amount of hexose sugars was similar in both cases, which indicated that in the early stages of germination little or no sugar had been absorbed. After this period the seedlings supplied with glucose contained considerably more hexose sugar, and also more ascorbic acid. Fig. 4 also shows that part of the glucose supplied to the seedlings was converted into fructose, since the latter was proportionately increased when glucose was supplied. It is possible that one or other, or both of these sugars may act as precursors of ascorbic acid, but the present data do not elucidate this point.

The results of a similar experiment in which glucose was supplied to seedlings growing in $(NH_4)_2SO_4$ are illustrated in Fig. 5. The addition of glucose slightly delayed the hydrolysis of sucrose and increased considerably the amount of hexose sugar, but the rise in ascorbic acid was relatively slight. The conversion to fructose of the absorbed glucose was not impaired under these conditions. A similar result was observed when NH_4Cl was substituted for $(NH_4)_2SO_4$. The fact that it was possible under these conditions to increase the hexose content of the seedlings to a value similar to that in seedlings grown in water, without at the same time appreciably increasing the ascorbic acid, suggested that the depressing effect of $(NH_4)_2SO_4$ or NH_4Cl cannot be due simply to a reduction in the amount of hexose sugars, or to a failure to convert glucose into fructose.

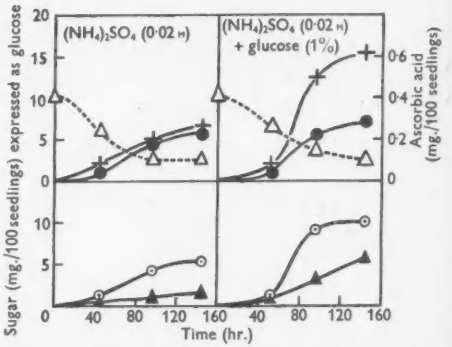


Fig. 5. The ascorbic acid and sugar content of cress seedlings germinated in presence of $(NH_4)_2SO_4$ and glucose; △—△, sucrose; +—+, hexose (T.R.S. – N.F.); ●—●, ascorbic acid; ▲—▲, fructose; ⊙—⊙, glucose.

When glucose was supplied to seedlings growing in solutions of Na salts which favour the synthesis of ascorbic acid, e.g. succinate or pyruvate, the increase in the hexose sugar was associated with a greater increase in ascorbic acid. The relationship between ascorbic acid and hexose formation in these and the foregoing experiments is shown more clearly in Fig. 6*A*, *B*. Here the ascorbic acid is plotted against the hexose; the curves all pass through the origin, but have gradients which appear to be determined by the nature of the salt used. The salts fall into three distinct groups: Na or K salts containing a utilizable anion give curves with the steepest gradients (group 1), water alone or ammonium salts containing a utilizable anion give curves with an intermediate gradient (group 2), whilst ammonium salts con-

taining a non-utilizable anion give curves in which the gradient is still further reduced (group 3).

Furthermore, when seedlings growing in these various salt solutions are supplied with glucose, the formation of ascorbic acid is increased by an amount

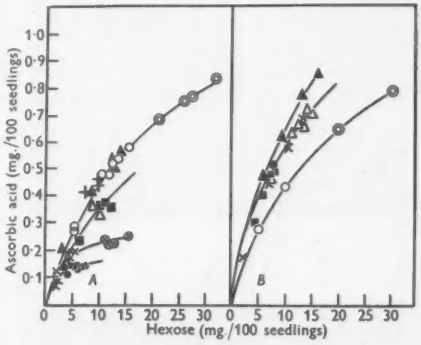


Fig. 6. The relation between ascorbic acid and hexose formation as affected by different salts. *A*, ○, water; ●, NH_4Cl (0·04M); ×, $(NH_4)_2SO_4$ (0·02M); ■, Na_2SO_4 (0·02M); △, NaCl (0·04M); +, NH_4HCO_3 (0·02M); ▲, ammonium succinate. *B*, ○, water (curve for comparison); △, sodium succinate (0·02M); ×, $KHCO_3$ (0·02M); ■, KNO_3 (0·04M); ▲, sodium pyruvate (0·04M). Ringed symbols represent values obtained when D-glucose was supplied in addition to salt.

which is determined by the gradient of the curve, the points corresponding falling on the extrapolated curve. Thus, with salts in group (1) the increased synthesis of ascorbic acid induced by increasing the hexose sugar of the seedling is relatively greater than with salts of group (2) and very much greater than with salts of group (3).

The simplest interpretation of such data is that ascorbic acid is formed directly from hexose, and that, as compared with seedlings grown in water, this conversion is stimulated by salts of the first group, is unaffected by salts of the second group and impaired by salts of the third.

As was pointed out in an earlier paper (Mapson & Cruickshank, 1947) these three classes of salts differentially affect the pH of the cell sap. Thus, compared with seedlings grown in water, salts in group (1) increase the pH of the cell sap, those in group (2) do not appreciably alter it, whilst those in group (3) reduce it. These changes in pH have been studied in more detail in this work. The data (Fig. 7) show the changes in pH during germination of seedlings grown in water, and in solutions of salts representative of the three groups mentioned above. The gradients of the curves showing the relation between ascorbic acid and hexose sugars form a well-defined series in which the steepest are associated with high pH and the less steep with low pH of the cell sap. In all experiments there was a drift in the pH of the cell sap to more alkaline values as growth of the seedling increased, but this did not appear to alter the quantitative relation between ascorbic acid and hexose.

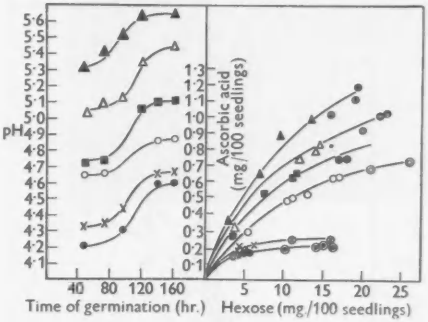


Fig. 7. The effect of different salts on the pH of the cell sap and the relation between ascorbic acid and hexose in cress seedlings; temp. 20°; ●, NH_4Cl (0·04M); ×, $(NH_4)_2SO_4$ (0·02M); ○, water; ■, ammonium succinate (0·02M); △, sodium succinate (0·02M); ▲, sodium pyruvate (0·04M). Ringed symbols represent values obtained when D-glucose was supplied in addition to the salt.

Sugars other than glucose

Since the foregoing evidence supported the view that ascorbic acid is formed from hexose sugars, it was considered desirable to ascertain whether other sugars could similarly be converted into ascorbic acid. The effect of supplying pentoses and hexoses other than glucose was therefore investigated. L-Arabinose, D-xylose and D-ribose, in concentrations of 0·5 and 1 % were without effect on ascorbic acid, although quite considerable amounts were absorbed by the seedling, as shown by the increase in the non-fermentable sugar fraction. Sucrose, D-fructose and D-galactose each increased the synthesis to about the same extent as glucose. On the other hand, D-mannose depressed synthesis and also adversely affected growth, its influence being proportional to the concentration employed.

Mannose supplied in combination with other sugars

The marked inhibition of the synthesis of ascorbic acid induced by supplying D-mannose appeared to be of sufficient importance to warrant further study, and the following experiment was carried out. The results (see Fig. 8) illustrate the effect on the synthesis of ascorbic acid of supplying different concentrations of glucose in combination with 0·5 % of mannose. It will be seen that the production of

ascorbic acid was not affected during the first 50 hr. Subsequently the inhibitory effect of mannose decreased progressively as the concentration of glucose increased. Similar results were obtained when galactose or fructose was substituted for glucose.

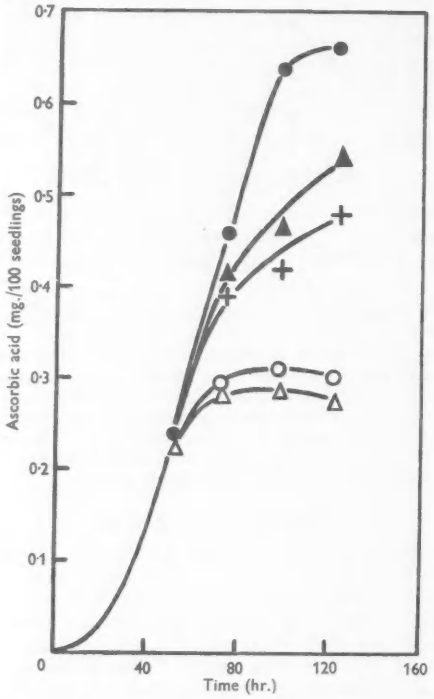


Fig. 8. Effect of mannose together with glucose; ●—●, water; ▲—▲, mannose (0·5%) + glucose (1%); +—+, mannose (0·5%) + glucose (0·5%); ○—○, mannose (0·5%) + glucose (0·25%); △—△, mannose (0·5%).

Changes in fat during germination

The poor development of hexose sugars in seedlings germinated in solution of salts like $(NH_4)_2SO_4$ prompted us to investigate whether such salts affected the production of these sugars by reason of their influence on the mobilization of the reserves of fat of the seedling. The data obtained, although throwing no further light on the main problem with which this paper is concerned, were nevertheless of sufficient interest to be included here.

Reference to Fig. 1 shows that hexose sugars are formed in the seedling during germination in amounts exceeding those which could be produced from the sucrose present in the seed. Since under the conditions of these experiments photosynthesis was precluded, such sugars must be derived mainly from the reserves of fat which the seed contains. The changes in the amount of fat present in seedlings grown in water and in $(NH_4)_2SO_4$ are shown in Fig. 9. During the first 48 hr. there was little or no reduction in the amount of fat, but, as growth proceeded, it steadily decreased. The rate of mobilization was more rapid in seedlings grown in water than in those grown in $(NH_4)_2SO_4$, the fat content at the end of 168 hr. falling from 57 mg. to 20 and 30 mg. respectively. On the assumption that the average

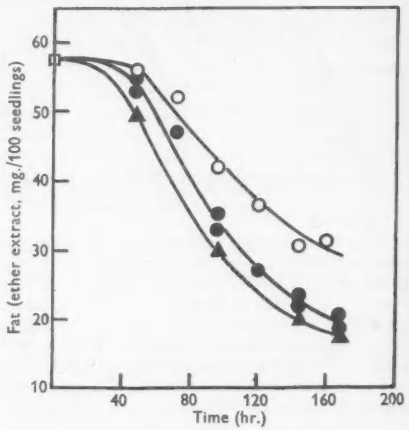
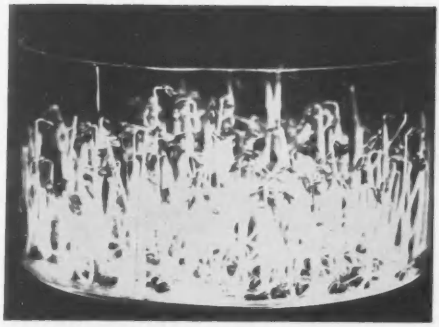


Fig. 9. Changes in fat content of seedlings during germination; □, dry seed; ○—○, seeds germinated in $(NH_4)_2SO_4$ (0·02 M); ●—●, seeds germinated in water; ▲—▲, seeds germinated in KNO_3 (0·02 M).

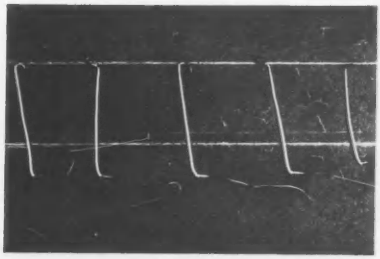
number of carbon atoms in the triglycerides is 20, and that these can be quantitatively converted to hexose, 10 mg. of fat (i.e. the maximum observed difference) could lead to the production of approximately 20 mg. of hexose sugar, which is much greater than the difference observed between the amount of hexose formed in seedlings grown in water and in $(NH_4)_2SO_4$. In those instances in which rather more hexose sugar was formed, e.g. in seedlings grown in KNO_3 or in water, there was some evidence that fat was mobilized rather more rapidly.

These results suggest that the decrease of hexose sugars in seedlings grown in $(NH_4)_2SO_4$ is partly, though not necessarily wholly, due to a reduced mobilization of fat.

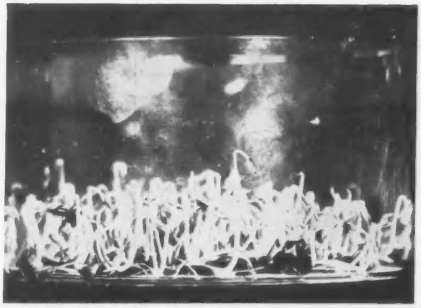
Acid value of fat. The acid value of the fat in the ungerminated seed was low, but as germination proceeded it increased considerably in the fat of seedlings grown in water and in all the solutions so far tested (Table 1). In those instances where the mobilization of fat was rather more rapid than in seedlings grown in water, e.g. when KNO_3 and sodium



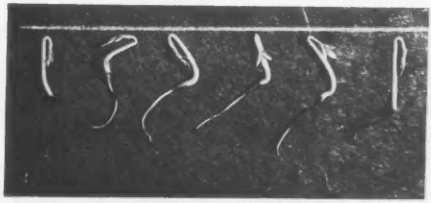
(*a*)



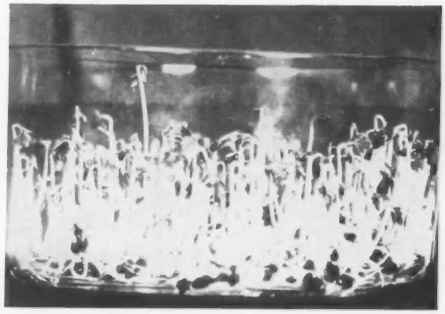
(*b*)



(*c*)



(*d*)



(*e*)

(*f*)

Seedlings germinated in water (*a*), (*b*); seedlings germinated in $(NH_4)_2SO_4$ (0·02 M) (*c*), (*d*); seedlings germinated in $(NH_4)_2SO_4$ (0·02 M) + sodium succinate (0·02 M) (*e*), (*f*). Photographs (*a*), (*c*) and (*e*) are pictures of seedlings germinated in jars and (*b*), (*d*) and (*f*) are photographs of separate seedlings to show more clearly the form of growth.

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succinate were supplied, the acid values were slightly higher. On the other hand, where the mobilization of fat was retarded, as when $(NH_4)_2SO_4$ was used, the acid values were slightly lower than when the seedlings were grown in water.

Iodine value of fat. The iodine values of the fat of seedlings grown in water, $(NH_4)_2SO_4$ and CH_3COOK, are also shown in Table 1. It will be seen that, although the iodine value of the fat at 48 hr. had dropped slightly as compared with that in the ungerminated seed, there was no evidence of any significant rise or fall during the later stages of growth. This is in contrast to the findings of Ivanow (1912), who observed that in poppy seedlings 8 days old the iodine value of the fat was 71·6, as compared with 140·2 in the fat of the ungerminated seedling. He also obtained similar results with linseed. This rapid fall in iodine value was attributed to the relatively rapid disappearance of the more highly unsaturated fatty acids. Miller (1912), in experiments on the sunflower, found that the iodine value of the fat of the hypocotyls and roots fell very considerably from the fourth to the fourteenth day of growth (from 117·7 to 48·3), whereas the iodine value of the fat from the cotyledons showed much less reduction during this period (from 125·5 to 111·7). The results of these two workers suggest that the unsaturated acids were preferentially utilized for conversion into hexose, but there was no clear evidence of this in our experiments.

Table 1. *Acid and iodine values of the fats of cress seedlings*

Duration of exp. (hr.)	Acid values. Fat of ungerminated seed, 1·6						Iodine values. Fat of ungerminated seed, 140		
	Exp. 1: treatment		Exp. 2: treatment		Exp. 3: treatment		Exp. 4: treatment		
	Water	$(NH_4)_2SO_4$ (0·02 M)	Water	KNO_3 (0·02 M)	Water	Sodium succinate (0·02 M)	Water	$(NH_4)_2SO_4$ (0·02 M)	CH_3COOK (0·02 M)
48	5·9	4·4	11·7	10·3	5·7	5·0	129·1	131·9	132·3
72	8·8	6·2	—	—	8·4	11·1	130·9	128·8	124·2
96	14·6	13·1	22·2	22·3	11·7	14·3	130·0	129·1	129·7
120	20·7	14·0	—	—	17·0	19·2	126·7	128·9	126·2
144	25·9	19·5	36·7	46·0	—	39·2	128·9	—	118·7
168	50·2	32·2	39·1	53·4	47·0	55·0	127·0	133·9	—

Influence of the nutrient medium on the form of growth of cress seedlings

Salts which depressed the synthesis of ascorbic acid, e.g. $(NH_4)_2SO_4$, NH_4Cl, $NH_2OH.HCl$, also influenced the form of growth (Pl. 1). Seedlings grown in water had erect and rather elongated hypocotyls, whereas in those grown in $(NH_4)_2SO_4$ the hypocotyls were bent, with a hooked appearance. When the sodium salts of succinic, pyruvic or acetic acids were supplied in addition to $(NH_4)_2SO_4$, the type of growth much more closely resembled that of seedlings grown in water. The reason for this is not clear. Evidently the salts do not operate by reason of their influence on the hexoses or ascorbic acid, because sodium succinate, for example, does not increase the former, and though it increases the latter, this can be done also by other means, e.g. by supplying ascorbic acid in combination with $(NH_4)_2SO_4$, without any resultant modification of the form of the hypocotyls.

When mannose was supplied, growth was very poor, and, as in the case of seedlings grown in $(NH_4)_2SO_4$, the hypocotyl was bent. Wachtel (1943), also using cress (cardamine) seedlings, has found that mannose inhibited growth.

DISCUSSION

Our results show that a close correlation exists between ascorbic acid and hexose sugars in the seedling, irrespective of whether it is germinated in water or in salt solutions which increase or decrease the synthesis of the vitamin. The simplest assumption is that hexose is converted to ascorbic acid, and the gradients of the curves expressing the relation between these constituents suggest that the conversion may be influenced by the nature of the salt present. On this hypothesis, Na or K salts containing a readily utilizable anion (e.g. HCO_3^-, pyruvate, succinate, etc.) favour the synthesis of ascorbic acid because they increase the conversion of hexose to ascorbic acid. Conversely the reduced synthesis of ascorbic acid in the presence of ammonium salts containing a utilizable anion, and the still poorer synthesis with ammonium salts containing a non-utilizable anion (e.g. SO_4^{--} or Cl^-) may be explained as being due to a decrease in the efficiency of conversion of hexose to ascorbic acid.

This interpretation of the experimental data is supported by the fact that when glucose is supplied to seedlings growing in any one salt solution, the increase in the ascorbic acid synthesized is quantitatively what would be expected by extrapolation of the curve relating ascorbic acid to hexose formation for that particular salt. Thus glucose supplied to the seedling behaves in the same way as hexoses derived from the seed reserves in relation to the formation of ascorbic acid.

The reason why these various salts should affect the conversion of hexose to ascorbic acid has still to be found. It is significant, however, that a correlation exists between the effect these salts have on the hexose/ascorbic acid relationship and their effect on the pH of the cell sap. Thus salts which induce a more alkaline pH of the cell sap favour the conversion of hexose to ascorbic acid and salts which depress the pH decrease the conversion. So far we have encountered no exception to this rule.

In this discussion we have assumed that the changes in ascorbic acid observed with the different treatments are due principally to an alteration in the synthetic activity of the seedling. It is possible, however, that other factors are operating. The concentration of ascorbic acid in the cell at any time may well be conditioned *inter alia* by two factors: (1) the rate of synthesis of ascorbic acid from a precursor such as hexose sugars, and (2) the rate of oxidation of ascorbic to dehydroascorbic acid. The small amount of ascorbic acid produced by seedlings growing in $(NH_4)_2SO_4$ could therefore be the result of either a defective synthetic mechanism or of an increase in the rate of oxidation to dehydroascorbic acid brought about by a general rise in the oxidation potential of the system. The further possibility that both factors may be operative cannot be excluded. Similarly, the large amounts of ascorbic acid found in the presence of certain K or Na salts may well be due to a reversal of these effects. It is hoped to present evidence on this subject in a further communication.

It is of interest that the synthesis of ascorbic acid was greatly reduced when D-mannose was supplied. This is in contrast to the observations of Tadokoro & Nisida (1940), who found that the ascorbic acid content of maize and soya bean grown in a nutrient solution was increased by the addition of mannose. We have already referred to the work of Ray (1934), whose results agree with those of Tadokoro. It should be noted that these two workers were using plants belonging to the Gramineae and Leguminosae, while we used a cruciferous plant.

The inhibitory effect of D-mannose on the synthesis of ascorbic acid was diminished by the presence of D-glucose, D-galactose and D-fructose, sugars which, when supplied alone, favoured the synthesis of ascorbic acid. It is possible, therefore, that we have here an instance of inhibition of the competitive rather than the non-competitive type, that is, the glucose and mannose are in competition for the surface of some enzyme associated with the synthesis of ascorbic acid. If this is so, then the contrast between the effect of D-glucose and D-galactose and that of D-mannose suggests that the stereochemical configuration of C–3 in sugars is important in determining whether these can be converted into ascorbic acid.

SUMMARY

1. In cress seedlings germinated in the presence of various salt solutions in the dark, a close correlation was generally found between the amount of ascorbic acid and hexose sugars synthesized.

2. Salts, particularly those of ammonium, which contained an anion not readily utilized, reduced both these constituents. Sodium and potassium salts containing an anion readily utilized (e.g. bicarbonate pyruvate, succinate) increased the ascorbic acid but not the hexoses.

3. Solutions of D-glucose, D-galactose and D-fructose alone or combined with various salt solutions increased the hexose sugars. Ascorbic acid was also increased, to an extent determined by the effect produced on the hexose/ascorbic acid relationship by the individual salts. There was no evidence that the hexose supplied behaves differently from that derived from the seed reserves.

4. It is suggested that hexose is converted into ascorbic acid and that the efficiency of the conversion is influenced by the nature of the salt present, possibly through its effect on the pH of the cell sap. Salts which raise the pH increase, and those which lower it decrease, the efficiency of conversion.

5. The rate of mobilization of fat was slower in seedlings grown in ammonium sulphate than in those grown in water, and may partly account for the lowered formation of sugars in the former.

6. In contrast to the other hexose sugars, D-mannose depressed the synthesis of ascorbic acid, but its action could be diminished by supplying in addition D-glucose, D-fructose or D-galactose.

7. The form of growth of the seedlings was modified in the presence of ammonium sulphate or ammonium chloride, but became more nearly normal when such salts were supplied with a sodium or potassium salt containing a utilizable anion.

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Studies on Metabolic Products of the *Penicillium luteum* Series

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Considerable attention has been devoted to the polysaccharide metabolic products of *Penicillium luteum* Zukal. Raistrick & Rintoul (1931) isolated the mucilaginous polysaccharide, luteic acid, which was shown to be a malonylpolyglucose. Alkaline or mild acidic hydrolysis of luteic acid yielded the acid-free polysaccharide, luteose, which was constituted mainly of β-glucose units linked through the 1:6-positions (Anderson, Haworth, Raistrick & Stacey, 1939). Luteic acid was elaborated by *P. luteum* when grown on a number of different hexoses, pentoses or glycerol as sole source of carbon (Birkinshaw & Raistrick, 1933). Later, Anderson & Raistrick (1936) showed that although luteic acid was the main metabolic product of *P. luteum* Zukal, other laevorotatory polysaccharides, built up of mannose, galactose and fructose units, were produced in proportions varying with the age of the culture. Anderson *et al.* (1939) isolated a laevorotatory polysaccharide which gave D-galactose and D-mannose on hydrolysis and for which the name galuteose was proposed.

The present work is concerned with the isolation of two hitherto undescribed polysaccharides, the properties and component sugar residues of which have been investigated. A study of the sedimentation of the two new polysaccharides in the ultracentrifuge is presented in the following paper (Ogston, 1949).

EXPERIMENTAL

Strains of Penicillium luteum

Six strains of *P. luteum* were used; they are distinguished by their laboratory catalogue numbers. The strains were broadly similar in their cultural characteristics and morphological appearance and clearly belong to the *P. luteum* series. Strains F 6, 39, 46, 51 and 52 agreed closely with Thom's (1930) description of *P. luteum* Sopp and F 231 was a conidial non-ascosporic form closely corresponding to *P. luteum* Zukal. Strains F 6, 39, 46, 51 and 52 give broadly spreading floccose colonies on Czapek-Dox agar which were yellow at the margin and slowly developed greyish green conidial areas in the centre. The reverse was orange to red in colour. On beer-wort agar, broadly spreading thick floccose felts developed with zonate bands of yellow, pink and green conidial areas; the reverse was orange to red. On nutrient gelatin medium, broadly spreading mainly white floccose, buckled colonies developed with some pink zones and green conidial areas. The gelatin was liquefied and became red in colour. These strains were isolated in the laboratory, F 39 from mouldy cake, F 46 from a solution of sodium carboxymethylcellulose and F 51 and 52 from an industrial acetone recovery tower. Strain F 231 was purchased from the National Collection in January 1947 as *P. luteum* Zukal N.C.T.C. 585. On Czapek-Dox agar it formed broadly spreading mainly white floccose colonies with marginal green conidial zones; the reverse was colourless. Similar colonies were formed on nutrient gelatin medium, which was liquefied and remained colourless. In all the above strains the sterigmata were acuminate, 10–11 × 2·5 μ. and conidia ovoid about 2·5 × 3·5 μ.

Cultural methods

The cultures were grown in glass culture vessels ('Glaxo' bottles) each containing 350 ml. of Czapek-Dox medium of the following composition: glucose, 50 g.; $NaNO_3$, 2·0 g.; K_2HPO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1 l. After sterilization by autoclaving, each vessel was sown with a spore suspension from 7-day-old beer-wort agar slopes. The cultures were incubated at 25° in darkness, and usually harvested when the glucose was virtually completely exhausted. Initial and final glucose concentrations were determined in samples of the culture filtrates by the Fehling-Soxhlet method in which cuprous oxide was determined iodometrically by the method of Shaffer & Hartmann (1921). Carbon was determined by the wet combustion method of Houghton (1945). Volatile neutral

compounds, volatile acids and non-volatile acids were determined by methods based on those of Birkinshaw & Raistrick (1931). Data based on single flask cultures of the six strains are summarized in Table 1, and show that from 11 to 54% of the glucose carbon fermented by the various strains was converted to soluble metabolic compounds in the filtrates. All the cultures produced acid during growth, but there was no evidence of the formation of significant quantities of volatile acids or volatile neutral compounds.

pH 4·92 and on electrometric titration (glass electrode) 25·0 ml. 0·1 N-NaOH were required to neutralize the filtrate to pH 7·0. Under corresponding conditions, luteic acid (5 g.) required 22·5 ml. N-NaOH for neutralization (Raistrick & Rintoul, 1931). The filtrate had a reducing value (R.V.) corresponding to 6·98 g./l. apparent glucose, which was equivalent to 32·8% of the crude polysaccharide (38·7% on an ash-free basis). The reducing value before hydrolysis was 3·3% as apparent glucose. The substance gave no reaction with iodine. A sample of the crude product (15·0 g.) in water (30 ml.) was dialysed in a collodion sac against six changes of distilled water for 66 hr. The polysaccharide was recovered by concentration *in vacuo* to 30 ml. and addition of ethanol to 66% concentration (w/w) obtained 4·39 g. (29·3%). The low recovery on dialysis suggested that the polysaccharide may have a comparatively low molecular weight and undergo slow diffusion. The dialysed product had an ash content of 2·2%.

Table 1. *Preliminary analysis of carbon compounds present in culture filtrates of strains of* Penicillium luteum

P. luteum Strain F	Time of growth (days)	pH Initial	pH Final	Carbon (g./l.) Initial	Carbon (g./l.) Final total	Carbon (g./l.) Final glucose	Carbon (g./l.) Volatile neutral compounds	Volatile acids (ml. N-acid/l.)	Non-volatile acids (g. Ca salt/l.)	Metabolic carbon compounds (% initial C)	Metabolic carbon compounds (% C fermented)
6	12	5·8	3·5	19·12	8·83	0·60	0·11	0·0	—	43·0	44·4
39	18	5·5	4·7	20·20	5·02	0·90	—	—	—	20·4	21·4
46	25	7·0	2·9	18·51	10·62	1·24	0·46	4·2	20·40	50·7	54·3
51	24	5·5	3·3	20·20	9·78	0·80	0·69	0·0	—	44·4	46·3
52	18	5·5	—	20·20	2·32	0·0	—	—	—	11·5	11·5
231	92	5·8	3·5	18·72	11·10	8·12	—	—	—	15·9	28·1

Isolation of polysaccharides

Preliminary experiments showed that the culture filtrates contained polysaccharides, specimens of which were isolated, as follows. Spores of each of the six strains were seeded into groups of ten flasks, containing 350 ml. of medium (initial glucose, 49·0 g./l.). At the completion of fermentation (Table 2) the culture filtrates were separated from the mycelial felts by filtration through fluted paper. The filtrates were evaporated *in vacuo* at low temperature to 0·1 vol. and the polysaccharides precipitated by addition of 4 vol. of ethanol. The polysaccharides separated out as heavy syrupy precipitates which were desiccated with difficulty by trituration with increasing strengths of ethanol. They were finally washed with absolute ethanol and ether, and dried *in vacuo* over P_2O_5. The yields and some of the properties of the crude products are summarized in Table 2.

Tollens's phloroglucinol test was negative indicating the absence of pentose residues. The naphthoresorcinol reaction for uronic acids was negative. A negative result was also obtained with Dische's (1947) carbazole reaction for uronic acids. The crude polysaccharide gave a positive reaction with the Seliwanoff resorcinol reagent, indicating the presence of ketose sugars.

The polysaccharides from the other strains in 100 ml. aqueous solution, adjusted to pH 2 with HCl, were freed from

Table 2. *Isolation and properties of crude polysaccharides from strains of* Penicillium luteum

Strain F	Culture filtrate: Time of incubation (days)	Culture filtrate: Final glucose (g./l.)	Culture filtrate: pH	Crude polysaccharide: Yield (g.)	Crude polysaccharide: Yield (as % of initial glucose)	Crude polysaccharide: Ash (%)	Crude polysaccharide: $[\alpha]_D$ (°)	Oxidation product: Substance and m.p.	Oxidation product: Yield (as % of polysaccharide)
6	21	1·0	3·93	18·10	10·6	15·2	+ 7·0	Mucic acid, 206°	22·8
39	21	1·4	3·11	34·43	20·1	13·8	+ 5·8	Mucic acid, 214°	17·9
46	21	1·3	2·97	30·68	17·9	11·7	+ 8·5	Mucic acid, 209–211°	12·6
51	22	0·6	3·02	41·64	24·3	10·3	+ 5·7	Mucic acid, 215°	11·0
52	22	1·6	3·10	18·54	10·8	15·0	0·0	Mucic acid absent	—
231	41	2·0	3·88	27·77	16·2	19·6	+12·9	Mucic acid absent	—

The properties of the crude polysaccharide from strain F 6 were examined in some detail. On ignition the substance gave 15·2% of colourless ash. Its specific rotation, $[\alpha]_D^{18°}$, was +7° in water (*c*, 1); crude luteic acid has $[\alpha]_D$ − 25 to − 30°. A sample of the polysaccharide (5·32 g.) was hydrolysed with N-H_2SO_4 (50 ml.) at 100° for 7·5 hr. An equivalent of saturated $Ba(OH)_2$ solution was added to the cooled hydrolysate, the mixture diluted to 250 ml. and filtered. The filtrate gave ash and low molecular impurities by dialysis in collodion sacs against repeated changes of distilled water until the dialysate was neutral (72 hr.). The dialysed polysaccharide solutions were evaporated *in vacuo* to about 10% concentration and fractionated by addition of increasing proportions of ethanol. Properties of the isolated fractions are summarized in Table 3. Acid equivalents were determined by titration of 0·02 g. of polysaccharide in 5 ml. water with 0·01 N-NaOH (CO_2 free)

Table 3. *Ethanolic fractionation of crude polysaccharides from strains of* Penicillium luteum

Source of polysaccharide, etc.	Fraction no.	Conc. of ethanol (%, w/w)	Weight (g.)	Acid equiv.	$[\alpha]_D^{20°}$ (°)
Strain F 6	(Insol.)	0	1·28	—	—
(25·02 g. dialysed; recovery, 14·50 g.; 57·9 %)	1	44·1	4·09	4730	—
	2	61·2	4·10	6060	+21·6
	3	70·3	2·62	530	−18·1
	4	76·0	1·06	450	−14·0
	(Sol.)	—	1·35	—	—
Strain F 39	1	50·8	2·30	1930	+ 5·0
(39·63 g. dialysed; recovery, 4·82 g.; 12·2 %)	2	56·5	1·78	2670	+11·0
	3	63·0	0·38	1240	− 7·2
	4	70·3	0·17	890	—
	5	76·1	0·04	—	—
	(Sol.)	—	0·15	—	—
Strain F 46	(Insol.)	0	1·66	—	—
(38·2 g. dialysed; recovery, 11·36 g.; 29·7 %)	1	39·3	0·90	—	—
	2	48·5	0·12	1260	—
	3	53·5	0·35	1790	+ 6·9
	4	54·8	0·73	2380	+14·4
	5	56·9	1·06	2850	+16·3
	6	59·8	0·45	730	−13·8
	7	69·1	0·98	1300	+ 1·3
	8	70·0	2·85	630	−14·8
	9	70·5	1·78	620	−19·2
	10	76·0	0·05	—	—
	(Sol.)	—	0·43	—	—
Strain F 51	1	47·0	4·32	1440	− 2·0
(35·6 g. dialysed; recovery, 6·56 g.; 18·4 %)	2	58·9	1·91	1180	−13·0
	3	63·4	0·09	—	—
	4	76·0	0·05	—	—
	(Sol.)	—	0·19	—	—
Strain F 52	1	54·5	8·99	2060	+17·6
(41·7 g. dialysed; recovery, 15·92 g.; 38·2 %)	2	58·0	2·53	600	−12·0
	3	63·6	2·60	440	−22·5
	4	70·2	1·20	440	−22·2
	5	76·0	0·21	440	−22·3
	(Sol.)	—	0·39	—	—
Strain F 231	1	52·0	0·17	3320	—
(19·0 g. dialysed; recovery, 1·68 g.; 8·8 %)	2	56·0	0·55	13000	+21·7
	3	65·7	0·64	6210	+13·9
	4	71·8	0·12	5470	—
	5	76·0	0·05	—	—
	(Sol.)	—	0·15	—	—

Table 4. *Properties of polysaccharide fractions (Strain* F 6)

Fraction	$[\alpha]_D^{20°}$ (°)	Ash (%)	pH of 1 % solution	R.V. (% apparent glucose) Before hydrolysis	R.V. (% apparent glucose) After hydrolysis	Mucic acid on oxidation (%)
1	—	0·55	4·21	1·50	86·0	38·3 (m.p. 210°)
2	+21·6	0·20	4·91	1·49	92·8	—
3	−18·1	0·0	3·20	3·81	84·3	3·3 (m.p. 210°)
4	−14·0	0·80	3·46	8·73	79·7	—

using phenolphthalein as indicator. Specific rotation data refer to aqueous solutions, $c = 1$. In the case of the fractions from strain F 6, reducing power was determined before and after hydrolysis and the products of nitric acid oxidation examined (Table 4). Reducing power before hydrolysis was determined with 0·02 g. quantities by Hanes's modification of the method of Somogyi (1937; see Hockenhull & Herbert, 1945). For determination of reducing power after hydrolysis, the polysaccharides (0·02 g.) were hydrolysed for 2 hr. at 100° with 2 N-HCl in sealed tubes, neutralized, and reducing sugars determined as above. Samples of polysaccharide (0·5 g.) were oxidized by the method of Kent & Tollens (1885), and the yield of mucic acid and its melting point recorded. (All melting points are corrected.)

Strain F 6 was shown to yield a mixture of at least two polysaccharides which were separated by ethanol fractionation into a dextrorotatory, neutral polysaccharide (F 1 and F 2) and a laevorotatory, acidic polysaccharide (F 3 and F 4). The former appeared to predominate in the crude polysaccharide. The dextrorotatory polysaccharide yielded mucic

acid (38·3%) on nitric acid oxidation, whereas the laevorotatory polysaccharide gave only an insignificant yield of the acid.

Strain F 39 gave a dextrorotatory, neutral polysaccharide, and in addition a very small amount of a laevorotatory polysaccharide.

Strain F 46 crude polysaccharide was shown to contain a dextrorotatory, neutral polysaccharide and a larger quantity of a laevorotatory, acidic polysaccharide.

Strain F 51 gave laevorotatory products only, which were weakly acidic (acid equivalent 1200).

Strain F 52 gave approximately equal quantities of a dextrorotatory, neutral polysaccharide and a laevorotatory, acidic polysaccharide ($[\alpha]_D^{20°}$ −22°, acid equivalent 440, corresponding to approximately one equivalent of acid for every three anhydrohexose units in the polysaccharide). Fraction 1 (7·8 g.) was refractionated between ethanolic concentrations of 50 and 60% (w/w) and shown to consist mainly of a dextrorotatory, neutral polysaccharide ($[\alpha]_D^{16°}$ +21·6, acid equivalent 3600, 4·27 g.). Nitric acid oxidation of fraction 3 ($[\alpha]_D$ −22·5°) gave oxalic acid (6·3%), and no mucic acid was detected.

Strain F 231 crude polysaccharide gave a very low recovery (8·8%) on dialysis. The product consisted of dextrorotatory, neutral polysaccharides. No laevorotatory substances were detected. Nitric acid oxidation of fraction 2 gave mucic acid (m.p. 207°, yield 43·9%). This fraction had R.V. before hydrolysis of 0·5% as apparent glucose. It corresponded closely in its properties to the dextrorotatory polysaccharide isolated from strain F 6.

Dextrorotatory polysaccharide from strain F 6

Isolation and properties of the polysaccharide. Preliminary experiments showed that the most satisfactory method of isolation of the pure dextro- and laevorotatory polysaccharides was by further ethanolic fractionation of the dextro- and laevo-rotatory fractions obtained in the preliminary ethanolic fractionation of the dialysed polysaccharide. The dextrorotatory polysaccharide was normally precipitated when the ethanolic concentration reached 50–60%. The following results were obtained in a typical experiment. The crude polysaccharide (40·7 g.) was dissolved in water and the insoluble fraction centrifuged off. The latter was twice extracted with water and the extracts combined with the main extract. The dried insoluble fraction weighed 3·57 g. The solution (150 ml.) was made acid to pH 2 with HCl and dialysed for 6 days against several changes of distilled water. The dialysed polysaccharide solution was concentrated *in vacuo* to 63 g. and ethanol added to 50% concentration (w/w). The precipitated polysaccharide was dried in the usual way; 8·18 g., acid equivalent 6000. The polysaccharide (6·93 g.) was dissolved in water (100 ml.) and divided into seven fractions by addition of increasing proportions of ethanol (Table 5). The acid equivalents and specific rotations of the fractions were determined. The main fractions (nos. 3–5, 5·25 g., 75·7% of the starting material) were practically identical, being virtually neutral and having specific rotations in the range +23·0 to +29·7°. Further ethanolic fractionation showed that fraction 3 consisted essentially of polysaccharide with specific rotation of +27·5.° Fractions with specific rotations in the range +27·5 to +29·7° were regarded as the pure dextrorotatory polysaccharide and were used in the experiments described below. The pure substance was ash-free; it contained 0·2–0·5% N, but was free from S and halogens.

Hydrolysis of polysaccharide. Preliminary experiments showed that liberation of reducing sugars from the polysaccharide on hydrolysis with 2N-H_2SO_4 at 100° was at a maximum after 8 hr. The polysaccharide (1·93 g.) in 2 N-H_2SO_4 (100 ml.) was hydrolysed under these conditions. The ice-cold hydrolysate was carefully neutralized with hot saturated $Ba(OH)_2$, a few drops of N-H_2SO_4 added and finally neutralized with an excess of pure $BaCO_3$. The precipitated $BaSO_4$ was centrifuged off and washed; the combined supernatant solution and washings were evaporated *in vacuo* to 100 ml. The neutralized hydrolysate had a total reducing value corresponding to 1·50 g. apparent glucose, i.e. 77·8% of the polysaccharide (1·56 g. as apparent galactose, i.e. 80·9% of the polysaccharide); its optical rotation $\alpha_D^{16°}$ was +1·065° (1 dm.) and specific rotation $[\alpha]_D^{16°}$ +71·0° (R.V. as glucose) and +68·2° (R.V. as galactose).

Identification of component sugars. Partition chromatograms on paper were made with samples of the neutralized hydrolysate described above (Partridge, 1946, 1948; Forsyth, 1948). The chromatograms were developed for 72 hr. at 20°. *n*-Butanol, ethanol, water (40, 10, 50% v/v) and *n*-butanol were used as solvents. D-Galactose, D-glucose, D-fructose and D-mannose were included as reference sugars.

Butanol-ethanol-water. The chromatograms gave only a single spot corresponding to galactose. Glucose and mannose

Table 5. *Homogeneity of dextrorotatory polysaccharide* (*Strain* F 6)

(Dextrorotatory fractions of dialysed polysaccharide were refractionated with ethanol and properties of the fractions determined.)

Fraction	Conc. of ethanol (%, w/w)	Weight (g.)	Acid equiv.	$[\alpha]_D^{20°}$ (°)	Nitrogen (%)
1	20·5	0·20	3100	—	—
2	49·3	0·40	6300	—	—
3	52·5	2·44	15000	+23·0	—
3*a**	—	—	10000	+27·5	0·47
4	54·8	1·95	14000	+28·9	—
5	57·7	0·86	24000	+29·7	0·18
6	69·0	0·26	7000	—	—
7	Mother liquor	0·23	940	—	—
		Recovery, 6·34 g., 91·5%			

* Fraction 3*a* was obtained by reprecipitation of fraction 3, three times between 49·5 and 53·0% alcoholic concentration.

were absent. The sensitive resorcinol reagent (Forsyth, 1948) was used to demonstrate the absence of ketose sugars.

Butanol. The chromatograms clearly showed the presence of galactose and the absence of glucose, mannose and fructose.

Quantitative determinations of galactose in the polysaccharide hydrolysate were carried out by the partition chromatographic technique described by Flood, Hirst & Jones (1947, 1948). The polysaccharide (10 mg.) and L-rhamnose (5 mg.) as reference sugar were hydrolysed with 0·4 ml. 2 N-H_2SO_4 in a sealed tube for 1 hr. at 100°. The neutralized hydrolysate was transferred to the chromatograms and developed with *n*-butanol, ethanol, water (40, 10, 50% v/v) for 72 hr. at 20°. Recoveries of galactose and rhamnose corresponding to 88% of the former in terms of the polysaccharide were obtained.

Yeast fermentation of polysaccharide hydrolysate. The chromatographic analyses described above showed that galactose was the main component sugar of the polysaccharide. The chromatograms demonstrated the absence of significant quantities of glucose, fructose and mannose. The absence of reducing sugars, other than galactose and those normally fermented by yeast, was confirmed by fermentation of the neutralized hydrolysate with a strain of *Saccharomyces carlsbergensis* Hansen (N.C.T.C. 742), which had been trained to ferment galactose. The organism was passaged three times in a medium containing 10 parts of hydrolysed lactose (5 g. lactose/l. hydrolysed with 0·5 N-HCl for 1·5 hr. at 100° and neutralized to pH 5–6) and 1 part of beer wort. The cells were washed twice with saline and suspended in the polysaccharide hydrolysate at 30° and pH 5–6 so that the cell density was about 80×10^6/ml. and fermentation was allowed to proceed for 96 hr. (Wise & Appling, 1944). A parallel fermentation with a similar concentration of D-galactose was carried out as a control. Reducing value determinations were made with 0·2 ml. samples of the supernatant solutions, freed from cells by centrifugation, by Hanes's modification of Somogyi's (1937; see Hockenhull & Herbert, 1945) method (Table 6). The

Table 6. *Fermentation of dextrorotatory polysaccharide hydrolysate by* Saccharomyces carlsbergensis

	Polysaccharide hydrolysate		D-Galactose	
Time (hr.)	Reducing value of fermentation mixture as apparent glucose (mg./100 ml.)	Residual R.V. (% of initial)	Reducing value of fermentation mixture as apparent glucose (mg./100 ml.)	Residual R.V. (% of initial)
0	383	100	243	100
72	9·5	2·5	6·5	2·7
96	8·4	2·2	2·3	0·9

galactose was rapidly fermented until less than 1% of the original reducing power remained after 96 hr. Similar results were obtained with the hydrolysate, which retained only 2·2% of the original reducing power after 96 hr. fermentation. It is concluded that less than 2·2% of the reducing substances (i.e. 1·8% of the polysaccharide) were present as sugars other than glucose, fructose, mannose and galactose.

Isolation of D-galactose and derivatives. A sample of neutralized hydrolysate corresponding to 2·435 g. polysaccharide was evaporated *in vacuo* to a thick syrup, glacial acetic acid (5 ml.) added to give an acetic acid concentration of about 90% and the mixture allowed to stand at 0°. After seeding with a trace of D-galactose a mass of colourless crystals was obtained which was filtered off and washed with glacial acetic acid; yield of crude D-galactose, 1·24 g. (51·0%). The crude product (m.p. 160–163°) was once recrystallized from 90% acetic acid, and almost pure D-galactose obtained, m.p. 163°, unchanged on admixture with authentic D-galactose, m.p. 166°. $[\alpha]_D^{18°}$ +113° after 5 min. → +79° after 19 hr.

D-Galactose methylphenylhydrazone. On treatment with 0·2 g. methylphenylhydrazine (asym. redistilled: b.p. 104° at 14 mm.) and 0·1 g. 50% acetic acid the sugar (0·25 g.) in 50% ethanol gave crystalline D-galactose methylphenylhydrazone (0·33 g.; 84% of the theoretical; Neuberg, 1907; Lüdtke, 1929). The crude product was recrystallized from hot pyridine, ethanol (1:1, v/v) mixture; after one to two crystallizations pure D-galactose methylphenylhydrazone, m.p. 189°, was obtained. The melting point was undepressed on admixture with the authentic hydrazone (m.p. 189°). (Found (Weiler and Strauss): C, 54·8; H, 7·1; N, 9·2. Calc. for $C_{13}H_{20}N_2O_5$: C, 54·9; H, 7·1; N, 9·9%.)

A further galactose determination on the neutralized hydrolysate was made by the methylphenylhydrazone method of Hirst, Jones & Woods (1947). A portion of the hydrolysate corresponding to 0·9637 g. of polysaccharide was treated with the methylphenylhydrazine reagent and gave 0·7767 g. galactose methylphenylhydrazone, which corresponds to 0·5358 g. galactose according to the formula of Hirst *et al.* (1947), i.e. to 55·6% of the polysaccharide.

The excess methylphenylhydrazine was removed as benzaldehyde methylphenylhydrazone from the mother liquor in the above determination as described by Morgan (1938). The volume of the residual solution was adjusted to 25 ml. The total reducing value corresponded to 0·0578 g. apparent glucose, i.e. 6·0% of the polysaccharide. The remainder (24 ml.) was evaporated to a syrup, dissolved in methanol (5 ml.) and treated with *p*-nitrophenylhydrazine (0·1 g.) as described by van Ekenstein & Blanksma (1903; Reclaire, 1908). No crystalline hydrazone was obtained under these conditions.

D-Galactose p-nitrophenylhydrazone. Neutralized hydrolysate equivalent to 2·275 g. polysaccharide was evaporated *in vacuo* to a thick syrup, which was dissolved in methanol (15 ml.) and heated with *p*-nitrophenylhydrazine (2·0 g.) for 10 min. on the water bath. A bright yellow microcrystalline solid soon separated on cooling. The product was filtered off on a sintered-glass crucible and washed with ice-cold ethanol and ether. On evaporation of the mother liquor and washings nearly to dryness *in vacuo* and addition of absolute methanol (10 ml.) further crops of the hydrazone were obtained. Yield of crude hydrazone 2·255 g., corresponding to 56·6% of the polysaccharide. The crude substance softened at 184° and melted with decomposition at 185°. It was recrystallized from boiling absolute ethanol, m.p. 194°. On admixture with authentic D-glucose *p*-nitrophenylhydrazone (m.p. 186°) the melting point was depressed to 184°. On admixture with authentic D-galactose *p*-nitrophenylhydrazone (m.p. 195°) the melting point was undepressed at 194°. (Found: C, 46·0; H, 5·3; N, 12·7. Calc. for $C_{12}H_{17}N_3O_7$: C, 45·7; H, 5·4; N, 13·3%.)

Acetylation of polysaccharide. Preliminary experiments were made with the crude polysaccharide to determine the optimal conditions for acetylation. The pure substance (0·545 g.) was dissolved in water (2 ml.); pyridine, redistilled

over KOH, b.p. 115° (25 ml.), was added and followed by redistilled acetic anhydride (25 ml.); the latter was added gradually so that the temperature of the mixture did not exceed 35°. The mixture was kept at 20° for 48 hr.; at this stage a sample gave no precipitate on pouring into ice-cold water. The solution was heated under reflux at 90° for 6 hr., cooled, and poured into 500 ml. of ice-cold water giving an immediate precipitate of almost colourless acetyl polysaccharide. The latter was filtered off, washed free from acid with water and dried *in vacuo* over P_2O_5. Yield, 0·690 g., i.e. 71·2% of the theoretical yield of triacetyl compound. Fractionation of the acetyl derivative showed that it was homogeneous. The crude substance (0·531 g.) was dissolved in $CHCl_3$ (15 ml.) and three times reprecipitated by addition of 3 vol. ether. The third precipitate was redissolved in $CHCl_3$ (10 ml.) and fractionated by addition of increasing proportions of ether. The main fractions were precipitated by 59% (v/v) and 75% (v/v) of ether, respectively; their weights were 0·213 and 0·120 g., total recovery 0·405 g. (76·3%). The specific rotations of the fractions were respectively, $[\alpha]_D^{17°}$ +38·3° and +44·1° in $CHCl_3$ (*c*, 0·4) and their acetyl contents, 46·2 and 49·2% (theory for triacetyl hexosan, 44·8%).

Benzoylation of polysaccharide. The polysaccharide (1·04 g.) was benzoylated by the Schotten-Baumann method. The crude benzoylated product (1·66 g., 54·5% of the theoretical for the tribenzoyl hexosan) separated almost immediately as an almost colourless precipitate. It was soluble in acetone and $CHCl_3$, but insoluble in ether and water. After fractional precipitation from $CHCl_3$ solution with ether the main fraction had a specific rotation of $[\alpha]_D^{18°}$ +18° in $CHCl_3$ (*c*, 0·6).

Laevorotatory polysaccharide from strain F 6

Isolation and properties of the polysaccharide. After removal of the crude dextrorotatory polysaccharide from solutions of the crude dialysed polysaccharide by addition of ethanol to 50–60% concentration, the mother liquor consisted predominantly of laevorotatory polysaccharide. A portion of crude laevorotatory polysaccharide precipitated from these mother liquors by increasing the ethanolic concentration to 80% (w/w) (9·68 g., $[\alpha]_D^{16°}$ −2·1°, acid equivalent 770) was redissolved in water (200 ml.), centrifuged to remove insoluble material and the clear, pale golden-brown coloured supernatant solution divided into six fractions by addition of increasing concentrations of ethanol. (The insoluble fraction formed a thick viscous paste on addition of water, but did not dissolve. It was readily soluble in N-NaOH and slowly soluble in 2N-Na_2CO_3, but insoluble in acids. No precipitate was obtained on acidification of the alkaline solution.) After removal of the first ethanolic precipitate (51·5% ethanol), the supernatant solution was decolorized by treatment with animal charcoal (2 g.) at 20° for 18 hr. The charcoal was centrifuged off and the ethanolic fractionation continued. After precipitation and centrifugation of fraction 3, the opalescent mother liquor was evaporated *in vacuo* to 35 g. and fractionation continued by addition of sufficient ethanol (70 ml., 61·4%) to produce an appreciable further precipitate. The fractions were precipitated as clear, colourless syrups which were desiccated with difficulty by treatment with increasing concentrations of ethanol; they were finally washed with ether (dried over Na) and dried *in vacuo* over P_2O_5. The yields and properties of the fractions are summarized in Table 7. The results showed that the main laevorotatory fractions were practically identical, having specific rotations in the range −13° to −17° and acid equivalents of 680–825 (mean 765, which corresponds to approximately one acidic group per five anhydrohexose units).

Table 7. *Homogeneity of laevorotatory polysaccharide (Strain* F 6)

(Laevorotatory fractions of dialysed polysaccharide were refractionated with ethanol and properties of the fractions determined.)

Fraction no.	Conc. of ethanol (%, w/w)	Weight (g.)	Acid equiv.	$[\alpha]_D^{18°}$ (°)
Insol.	0	1·294	941	−5·0 (in 0·1 N-NaOH)
1	51·5	0·183	1580	—
2	59·0	2·713	1296	+ 5·8
3	68·2	2·962	793	−10·4
3*a**	—	—	825	−13·3
4	61·4	0·421	682	−16·9
5	70·3	0·425	790	−14·5
6	75·9	0·088	971	—
Mother liquor	—	0·392	—	—
		Recovery, 8·478 g., 87·5%		

* Fraction 3*a* was obtained by fractionation of fraction 3, between 75 and 99% ethanol concentration (w/w).

The polysaccharide (10 mg.) gave no reaction for ketose sugars in the Seliwanoff test after 15 min. at 100°; under the same conditions fructose (2 mg.) gave a definite pink coloration after 2–5 min. On hydrolysis with N-H_2SO_4 (0·0577 g. polysaccharide in 25 ml.) at 100°, the initial reducing value of 5·3% as apparent glucose rapidly increased to a maximum of 67·8% as apparent glucose after 3 hr., and thereafter decreased at an approximately linear rate, reaching 60·0% after 6 hr. A sample of polysaccharide (0·4984 g.) was dissolved in 100 ml. N-H_2SO_4 and hydrolysed under these conditions. The hydrolysate (5 ml.) was treated with the equivalent of $Ba(OH)_2$ (12·9 ml., 0·387 N) and the precipitate of $BaSO_4$ centrifuged off. The supernatant solution (10 ml.) had pH 8·36 and required less than 0·1 ml. 0·1 N-H_2SO_4 for neutralization. It was clear that no acid was liberated on hydrolysis. The remainder of the hydrolysate (≡0·440 g. polysaccharide) was neutralized in the usual way and the neutralized hydrolysate diluted to 50 ml. Its reducing power was 69·6% (as apparent glucose) and its specific rotation on this basis was +47·8°. On heating at 100° for 30 min. with phenylhydrazine-HCl and sodium acetate, glucosazone was obtained. The solution of neutralized hydrolysate was used in the experiments described below.

Identification of component sugars. Chromatographic analyses of the laevorotatory polysaccharide hydrolysate were carried out as described above. The position of the isolated sugars on the chromatogram was detected by means of the ammoniacal $AgNO_3$ and resorcinol reagents.

Butanol-ethanol-water. The chromatograms gave only a single spot corresponding to glucose. Galactose, mannose and fructose were shown to be absent by use of the authentic sugars for reference purposes.

Quantitative determinations of glucose were made by the method of Flood *et al.* (1947, 1948). The polysaccharide (11·2 mg.) and L-rhamnose (5·0 mg.) as reference sugar were hydrolysed with 0·4 ml. 2N-H_2SO_4 in a sealed tube for 1 hr. at 100°. The chromatograms were developed with *n*-butanol, ethanol, water (40, 10, 50 v/v) for 72 hr. at 20°. The recoveries of glucose and rhamnose corresponded to 63·6% of glucose in the polysaccharide.

Acetylation of polysaccharide. The laevorotatory polysaccharide (0·529 g.) was dissolved in water (2 ml.) and pyridine (25 ml.), and acetic anhydride (25 ml.) added as described above. After 48 hr. at 20° a colourless precipitate had formed. This was filtered off, washed free from acid with water and dried *in vacuo* (0·588 g., i.e. 62·5% of the theoretical yield of *triacetylpolysaccharide*). The product had the unusual property of insolubility in $CHCl_3$, pyridine and acetone as well as in ether and water. (Found: C, 47·7; H, 5·7; acetyl, 30·7. $C_{12}H_{16}O_8$ ($C_6H_7O_5(CH_3CO)_3$) requires C, 50·0; H, 5·6; acetyl, 44·8%.)

The mother liquor was heated at 90° for 6 hr. and poured into ice-cold water. No further precipitate of acetylpolysaccharide was obtained.

Benzoylation of polysaccharide. The polysaccharide (0·55 g.) benzoylated normally by the Schotten-Baumann method giving 0·96 g. (59·6% of the theoretical yield) of crude benzoyl polysaccharide. The crude product (0·793 g.) was dissolved in $CHCl_3$ (5 ml.), twice reprecipitated by addition of 3 vol. ether, and redissolved in $CHCl_3$. The solution was fractionally precipitated with ether and shown to be homogeneous. The pure benzoyl polysaccharide had a specific rotation, $[\alpha]_D^{16°}$ +3·3° in $CHCl_3$ (*c*, 0·6).

Action of enzymes on the polysaccharides. Solutions of the dextro- and laevo-rotatory polysaccharides (2%, 1 ml.) were diluted with water (18·5 ml.) and a 1% solution of a malt amylase (mixture of α- and β-amylases) preparation (0·5 ml.) added; pH 4·6 and 30°. There was no increase of reducing power after 24 hr. incubation. The polysaccharides (2%, 1 ml.) were diluted with water (18·9 ml.) and invertase (yeast autolysate preparation) (0·1 ml.) was added; pH 4·6 and 30°. There was no increase of reducing power after 19 hr. incubation. The polysaccharides (2%, 1 ml.) were diluted with water (18·5 ml.) and 1% emulsin (British Drug Houses Ltd.; 0·5 ml.) added; pH 7 and 30°. There was no change in reducing power after 42 hr. incubation. The experiments showed that the polysaccharides were not attacked by α- or β-amylases, invertase or β-glucosidase.

Growth and polysaccharide production by Penicillium luteum F 6 *on various carbon sources*

A series of media was prepared, based on the Czapek-Dox formula, each containing one of the following substances as carbon source: glucose, galactose, lactose, maltose, soluble starch (2%), glycerol, sodium citrate cryst. (9·7%), sodium tartrate cryst. (6·4%), sodium acetate cryst. (3·8%), sucrose, xylose (1%), dulcitol (1%), mannitol (1%), sorbitol (1%), ethanol (2·5%) (concentration 5%, unless otherwise stated.) Three 'Glaxo' bottle cultures (350 ml. each) were sown with equal volumes of a spore suspension of *P. luteum* F6 for each carbon source. The initial pH of the media was pH 6–7. Observations on the growth rate of the fungus, the fall of reducing power in the sugar-containing media and pH changes are recorded in Table 8. The cultures were harvested after incubation at 25° for the periods stated in Table 8, the mycelium filtered off, dried and weighed. The culture filtrates were evaporated *in vacuo* to about 0·1 vol. and the ethanol-insoluble fraction, including polysaccharides, precipitated by addition of a slight excess (2–4 vol.) of ethanol. Samples (0·5 g.) of the crude 'polysaccharides' were oxidized with nitric acid by the Kent & Tollens (1885) method; the yields and melting points of the crude oxidation products are recorded in Table 8.

The mycelial growth on the galactose medium was heavier and more vigorous than that on any other medium; it differed significantly from that of the glucose medium, which was white and slimy with a light cream coloured culture filtrate, in its production of green conidial areas. The mycelium was reddish brown on its underside and the culture filtrate reddish brown in colour. Lactose also formed early (5 days) sporulation, but mycelial growth was slow and sparse. No growth took place on citrate or tartrate as sole source of carbon. The acetate medium gave rise to a trace of colourless submerged mycelium. Similar results were obtained with dulcitol, mannitol and sorbitol. Dulcitol and mannitol were recovered unchanged in yields of 95 and 85%, respectively, after incubation for 34 days. A thick syrupy product, which did not crystallize on standing, was obtained from the sorbitol medium; it was probably crude sorbitol. Moderately heavy growth of pink-coloured mycelium was obtained on the ethanol medium, which favoured the production of isolated colonies rather than a continuous felt. The observations of mycelial growth, recorded in columns 3–5 of Table 8, were visual estimates made during growth; they were not in every case supported by the mycelial weights finally obtained.

Reducing power determinations after 19 days' incubation showed that galactose was metabolized more rapidly than glucose; at this stage the mean galactose content was 0·60 g./100 ml. and that of glucose 0·89 g./100 ml. After incubation for 19 days the pH of the glucose medium fell to 2·63 and ultimately rose again to 3·90; the corresponding values for the galactose medium were 3·96 and 5·14.

The ethanol-precipitated fractions from the glucose, galactose, maltose, starch, glycerol, xylose and ethanol media were undoubtedly constituted mainly of polysaccharides. The product from the lactose medium (10·8 g., 20·6%) was the unchanged sugar; it reduced Fehling solution and its m.p. of 200° was undepressed on admixture with authentic lactose. On heating with acetic anhydride and sodium acetate it gave 59% of the theoretical yield of lactose octa-acetate, m.p. 95–100°, undepressed on admixture with authentic lactose octa-acetate. The sparse growth with this disaccharide, as compared with that on media containing its hydrolysis products, suggests that the fungus may not produce lactase. The bulk of the substrate was, however, probably metabolized by some other route since only 21% was recovered and the rotation ($\alpha_D^{17·5°}$ +1·39°, 1 dm.) of the culture filtrate was not in agreement with its reducing power (4·11 g./100 ml. as apparent lactose) on the assumption that the sugar was the main component. On addition of ethanol (2 vol.) to the concentrated culture filtrate a small immediate precipitate was obtained. On standing for a few hours the supernatant solution deposited lactose as a crystalline mass.

The culture filtrate from the galactose medium was deep red in colour. The coloured component was separated by extraction with $CHCl_3$ and on evaporation of the solvent orange-red crystalline needles were obtained (0·02 g., 0·04% of sugar fermented, m.p. 99–104°).

Table 8. *Growth of* Penicillium luteum F 6 *on various sources of carbon*

Carbon source	Conc. (%)	Mycelial growth after 9 days	21 days	27 days	Sugar conc. (% of final culture filtrate)	pH of culture filtrate	Cultures harvested after incubation (days)	Mycelium weight (g.)	Yield of polysaccharide (g.)	Percentage of substrate	Oxidation product	Yield of polysaccharide (%)	Reducing value as glucose: Before hydrolysis	After hydrolysis with 2 N-HCl in sealed tube, 2 hr.
Glucose	5·0	3	4	4	0·19*	3·90	27	6·10	3·64	6·93	Mucic acid, m.p. 202°	10·9	1·7	58·9
Galactose	5·0	4	4	4	0·15†	5·14	30	8·70	1·86	3·54	Mucic acid, m.p. 215°	23·3	1·7	28·9
Lactose	5·0	1	2	2	4·11†	7·13	30	1·04	Trace	—	—	—	55·7 (83·4§)	82·3
Maltose	5·0	3	4	4	0·34†	4·00	27	7·38	2·58	4·91	Mucic acid, m.p. 209°	20·0	2·1	50·3
Soluble starch	2·0	4	4	4	0·0	7·54	28	3·34	3·64	17·3	Mixture of mucic acid, m.p. 207° and oxalic acid, m.p. 99°	2·0	3·0	24·6
Glycerol	5·0	2	4	4	0·0	5·90	27	0·87	1·45	2·76	Mucic acid, m.p. 206°	7·6	1·1	26·2
Citrate ($Na_2C_6H_5O_7$ $5H_2O$)	9·7	0	0	0	—	—	27	0·0	0·0	0·0	—	—	—	—
Tartrate ($Na_2C_4H_4O_6.2H_2O$)	6·4	0	0	0	—	—	27	0·0	0·0	0·0	—	—	—	—
Acetate ($NaC_2H_3O_2$ $3H_2O$)	3·8	1	1	1	—	9·13	28	Trace	(2·01)	(5·04)	No mucic acid formed	—	0·7	2·9
Sucrose	5·0‡	2	2	3	2·85‡	3·22	34	2·50	1·58	3·01	Oxalic acid, m.p. 102°	13·3	1·0	48·7
Xylose	1·0	2	3	4	0·04*	3·84	27	2·55	1·41	13·4	Undetermined	14·7	1·7	14·9
Dulcitol	1·0	1	1	1	—	7·40	34	0·45	0·0	0·0	—	—	—	—
Mannitol	1·0	1	3	3	0·0	6·00	34	0·50	0·0	0·0	—	—	—	—
Sorbitol	1·0	1	1	1	—	7·08	34	0·60	0·0	0·0	Undetermined	—	—	—
Ethanol	2·5	2	2	3	0·0	5·66	34	1·11	1·47	5·60	Mucic acid, m.p. 196°	5·5	0·8	15·0

Mycelial growth key: no growth, 0; trace of growth, 1; fairly good growth, 2; good growth, 3; abundant growth, 4.
* As glucose. † As sugar specified. ‡ As invert sugar. § As anhydrous lactose.

After incubation for 28 days the soluble starch medium gave no I_2 reaction, showing that *P. luteum* excretes α-amylase into the medium. After concentration to 0·05 vol. the culture filtrate was non-viscous. The yield of polysaccharide (17·3%) and presence of mucic acid in its oxidation products showed that the starch had been metabolized by the fungus.

The culture filtrate from the acetate medium (pH 9·13) required 10 ml. N-HCl for neutralization to pH 6·93. The filtrate had no detectable rotation in a 1 dm. tube. The ethanolic precipitated fraction was non-polysaccharide in character; it gave a negative Molisch reaction and did not reduce Fehling's solution.

Growth and fermentation of the sucrose medium was poor as compared with the media containing the monosaccharides and maltose. After 34 days, 2·85 g./100 ml., i.e. 57% of the initial sucrose, remained unfermented.

Isolation of mucic acid (melting point undepressed on admixture with authentic mucic acid) on oxidation of the polysaccharide fractions from the glucose, galactose, maltose, starch, glycerol, and ethanol media showed that these substrates as sole sources of carbon were metabolized by *P. luteum* with the production of galactose-containing polysaccharides.

Growth of Penicillium luteum F 6 *on the dextro- and laevo-rotatory polysaccharides as sole sources of carbon*

Dextrorotatory polysaccharide. A medium based on the Czapek-Dox formula, containing the dextrorotatory polysaccharide (1%) as the sole source of carbon was sterilized in two portions of 10 ml. in conical flasks (50 ml.) by intermittent steaming at 100° on 3 successive days. The medium was inoculated with a weak spore suspension of *P. luteum* F 6, precautions being taken to avoid removal of nutrients from the agar slope in transferring the spores. The cultures were incubated at 25°. (Control cultures with medium containing salts, but no added carbohydrate, gave only traces of mycelial growth after 37 days.) After incubation for 37 days the polysaccharide medium contained only a trace of imponderable, semi-submerged mycelium.

Laevorotatory polysaccharide. A medium corresponding to that described above was prepared; it contained the laevorotatory polysaccharide (0·5%) as sole source of carbon. Nine portions of the medium (10 ml. each) were inoculated. After incubation for 5 days there was slight superficial growth with green conidial areas, and after 37 days the medium was covered with a continuous felt of colourless slimy mycelium, which was harvested, dried and weighed. The yield of mycelium (0·0994 g., i.e. 1·105 g./l. medium) was of the same order as that obtained from media containing lactose (5%), glycerol (5%), or ethanol (2·5%), Table 8. The combined culture filtrate slightly reduced Fehling solution. It had no detectable optical rotation.

Influence of Penicillium luteum *polysaccharides on aggregation of soil particles*

Geoghegan & Brian (1948*a*, *b*) showed that certain bacterial and mould polysaccharides had an aggregating effect on soil particles. The crude polysaccharide from *P. luteum* F 6, described in Table 2, had a definite aggregating effect, although less than that of levans and dextrans (Geoghegan & Brian, 1948*b*). The crude polysaccharides from strains nos. F 39, 46, 51, 52 and 231 and the pure dextro- and laevorotatory polysaccharides of strain F 6 were tested for soil aggregating properties (Table 9). The products from strains F 46 and 51 had slight aggregating effects, although small compared with the crude product from strain F 6 or levan. The pure dextro- and laevo-rotatory polysaccharides had much lower activities than the crude product from which they were obtained by alcoholic fractionation.

Table 9. *Influence of* Penicillium luteum *polysaccharides on aggregation of soil particles*

Polysaccharide	Percentage aggregation when 0·25% of the polysaccharide was applied to soil
Crude products:	
Strain F 6*	50·1
Strain F 39	1·8
Strain F 46	4·3
Strain F 51	14·4
Strain F 52	1·3
Strain F 231	1·6
Dextrorotatory polysaccharide, F 6	5·3
Laevorotatory polysaccharide, F 6	23·6
Levan†	90·9
Control	1·3

* Geoghegan & Brian (1948*b*).
† Geoghegan & Brian (1948*a*).

DISCUSSION

It is clear from the properties of the dextro- and laevo-rotatory polysaccharides that they are different from the *P. luteum* products hitherto described. The most important properties are summarized in Table 10 for comparison with those of luteic acid, luteose and galuteose. Anderson & Raistrick (1936) reported the formation in the metabolic products of *P. luteum* Zukal of laevorotatory polysaccharides, built up of mannose, galactose or fructose units in proportions varying with the age of the culture. Mannose was isolated as the phenylhydrazone (yield, 5%) from some of the crude fractions from our strain F 6 and fructose was detected by partition chromatography, but no pure polysaccharides containing these sugars were isolated. It is concluded that glucose and galactose are the predominant units for polysaccharide synthesis by *P. luteum*. The fungus synthesized a glucose-containing polysaccharide from glucose, fructose, mannose, xylose, arabinose and glycerol as sole sources of carbon (Birkinshaw & Raistrick, 1933) and a galactose-containing polysaccharide from glucose, galactose, maltose, starch, glycerol, and ethanol, but not from acetate, citrate, tartrate, sucrose, dulcitol, mannitol or sorbitol as sole sources of carbon.

The chemical evidence described above supports the conclusion that the polysaccharides are chemical entities. The ultracentrifugal data presented in the

Table 10. *Properties of* Penicillium luteum *polysaccharides*

Property	Luteic acid (Raistrick & Rintoul, 1931)	Luteose (Raistrick & Rintoul, 1931)	Galuteose (Anderson *et al.* 1939)	Dextrorotatory	Laevorotatory
Specific rotation, $[\alpha]_{5461}$ (°)	−47 (as Na salt)	−46·4	—	—	—
Specific rotation, $[\alpha]_D$ (°)	—	—	−25	+29	−16
Acid equivalent	434·7	Neutral	—	Neutral	800
Component units (100 g.):					
D-Glucose (g.)	83	100	—	0	64
D-Galactose (g.)	—	—	66	88	0
D-Mannose (g.)	—	—	20	0	0
Malonic acid (g.)	23·4	—	—	—	—
Viscosity of aqueous solution	Viscous	Viscous	Non-viscous	Non-viscous	Non-viscous

following paper (Ogston, 1949) shows, however, that they are polydisperse. The dextrorotatory polysaccharide consists of components of molecular weight of approximately 50,000 to 200,000 and the laevorotatory polysaccharide of components with a mean molecular weight of 15,000. The behaviour of the organism in producing a series of polysaccharides indistinguishable in their chemical properties, but of different molecular complexity, may be related to its power of producing a range of polysaccharide products according to the period of incubation as reported by Anderson & Raistrick (1936).

Stacey (1947) recorded that the biological function of the extracellular mould polysaccharide is unknown, it being generally assumed to act as a reserve carbohydrate. This may be the case with the laevorotatory polysaccharide which sustained about the same amount of mycelial growth as media containing lactose, glycerol or ethanol as sole sources of carbon. The dextrorotatory polysaccharide was not, however, attacked to any significant extent.

P. luteum is common in soils. The marked effect of the crude polysaccharide from strain F 6 on the aggregation of soil particles is of interest. The fungus may take part in producing crumb structure in soil (Geoghegan, 1947). The polysaccharides are hygroscopic and the difficulty of desiccation of their aqueous solutions has been noted; the latter may be of some biological significance to the fungus in delaying desiccation in soil.

SUMMARY

1. Isolation of two new polysaccharides from strains of *Penicillium luteum* series, grown on Czapek-Dox glucose medium, by ethanolic fractionation of dialysed culture filtrates is described.

2. The dextrorotatory neutral polysaccharide, $[\alpha]_D^{20°}+29°$ in water (*c*, 1), was non-viscous in aqueous solution. D-Galactose (88%) was the main component sugar; glucose, mannose and fructose were absent. The acetyl and benzoyl derivatives had specific rotations of $[\alpha]_D^{17°}+41°$ in chloroform (*c*, 0·4) and $[\alpha]_D^{18°}+18°$ in chloroform (*c*, 0·6), respectively.

3. The laevorotatory polysaccharide, $[\alpha]_D^{18°}-16°$ in water (*c*, 1), was acidic (acid equivalent, 800). D-Glucose (64%) was the main component sugar; galactose, mannose and fructose were absent. The acetyl polysaccharide was insoluble in the common organic solvents. The benzoyl derivative had a specific rotation of $[\alpha]_D^{16°}+3·3°$ in chloroform (*c*, 0·6).

4. Galactose-containing polysaccharide was synthesized by the mould from glucose, galactose, maltose, starch, glycerol, and ethanol but not from acetate, citrate, tartrate, sucrose, dulcitol, mannitol or sorbitol as sole sources of carbon.

Our thanks are due to Dr M. J. Geoghegan for carrying out the soil aggregation experiments.

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The Sedimentation and Diffusion of Polysaccharides from *Penicillium luteum*; Interpretation of the Results Obtained from Polydisperse Material in the Gouy Diffusiometer

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Samples of the dextro- and laevo-rotatory polysaccharides, prepared from *Penicillium luteum* by Freeman & Macpherson (1949), were submitted for the examination of their ultracentrifugal sedimentation and diffusion.

EXPERIMENTAL

The solid materials were dissolved in, and thoroughly dialysed against, buffer of composition: NaCl, 0·2 M; KH_2PO_4, 0·027 M; Na_2HPO_4, 0·027 M. The final concentrations were about 1 g./100 ml. and were estimated by refractometry against the buffer.

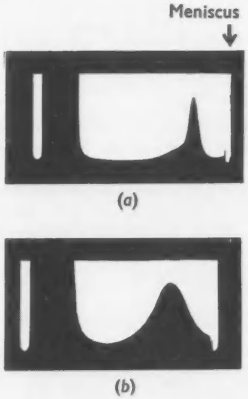

Fig. 1. Sedimentation diagrams: (*a*) dextrorotatory polysaccharide 37 min. after reaching full speed; (*b*) laevorotatory polysaccharide 80 min. after reaching full speed (60,000 rev./min.).

Sedimentation measurements. Sedimentation was observed in a Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938), using the standard conditions of running recommended by Cecil & Ogston (1948).

Both samples proved to be polydisperse. The dextrorotatory material (Fig. 1*a*) was composed of two main fractions: a more slowly sedimenting fraction which appeared to be homogeneous and a more rapidly sedimenting fraction which was heterogeneous. The sedimentation constant of the faster material and the relative proportions of the two fractions could not be determined accurately because the boundaries were not clearly resolved. However, separation of the schlieren curve into two more or less symmetrical parts (Fig. 2) gave an approximate estimate of the amounts of the two components. The combined boundary represented only 0·86 of the total refracting material.

The laevorotatory material (Fig. 1*b*) gave a single boundary, the thickness of which showed that the polysaccharide was polydisperse; it consisted of material sedimenting over a range of rates, symmetrically distributed about a mean value. Integration of this boundary showed that it represented only 0·64 of the total refracting material; however, this value may be too low, because the lower limit of the boundary appeared to be reaching the bottom of the cell before its upper limit had fully left the meniscus.

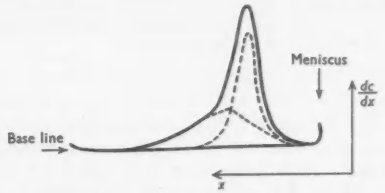

Fig. 2. Tracing from sedimentation diagram (full line) of dextrorotatory polysaccharide, 47 min. after reaching full speed (60,000 rev./min.), to show analysis into components (broken lines); gradient of concentration dc/dx against position in cell, x.

The results are given in Table 1.

Diffusion measurements. Diffusion was measured by means of the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). Ogston (1949) has shown that this method can be used to determine the diffusion constants of a mixture of two

Table 1. *Data obtained from ultracentrifugal sedimentation*

Material	Component	Fraction of total refracting material in ultracentrifuge diagram	Proportion of component in diagram	$\bar{S}_{20(\text{corr.})} \times 10^{13}$
Dextrorotatory	(1) Monodisperse	0·86	0·43	4·13
	(2) Polydisperse		0·57	5·2 (approx.)
Laevorotatory	Polydisperse	0·64	—	1·79

Table 2. *Data obtained from diffusion*

Material	Component	Proportion of component	$D_{20(\text{corr.})} \times 10^{7}$	Approx. mol. wt.
Dextrorotatory	(1) Monodisperse	0·27	5·5	50,000
	(2) Polydisperse	0·73	1·9	170,000
Laveorotatory	Polydisperse	—	Av. 8·32	15,000

homogeneous components. Provided that one of the components is present only in small amount, the error introduced by its polydispersity into the estimate of the diffusion constant of a homogeneous main component is small; however, if, as in the case of the dextrorotatory polysaccharide, a main component is polydisperse, considerable errors are introduced into the estimates of the diffusion constants of both components and of their proportions. The values quoted in Table 2 are, therefore, only rough and it is not surprising that the proportion of the components estimated from the diffusion data differs from that obtained from the sedimentation diagram (Table 1).

Any attempt to analyse the diffusion data given by a highly polydisperse material, such as the laevorotatory polysaccharide, in terms of two homogeneous components, is of little value and could do no more than indicate the range of polydispersity. It would be more useful to estimate the mean diffusion constant, for comparison with the mean sedimentation constant. However, the mean diffusion constant which is obtained from the movement of the outermost interference band by the Gouy method is not the weighted mean diffusion constant, but is given by

$$\frac{1}{\sqrt{\bar{D}}} = S\frac{\alpha_i}{\sqrt{D_i}},$$

where α_i is the fraction of material having diffusion constant D_i. Use of this value, together with the mean sedimentation constant, to calculate the molecular weight would yield a rather curious mean value. A method has therefore been found, and is described below, for obtaining the arithmetic-mean diffusion constant from the Gouy data. The result of this calculation is given in Table 2.

THEORETICAL

Method of obtaining an arithmetic-mean diffusion constant by the Gouy method

Where the diffusion boundary is made up of a range of superimposed Gaussian boundaries, if α_i is the fraction of the total refractive increment due to each component i having diffusion constant D_i and if v is the total refractive increment (expressed as numbers of wavelengths of phase difference), then the phase difference r of light passing through the boundary at distances $+x$ and $-x$ from its centre and the angular deflexion θ_r of such light at time t are given by

$$r = \frac{2v}{\sqrt{\pi}} S\{\alpha_i f(z_i)\},$$

where

$$f(z_i) = \int_0^{z_i} e^{-z^2}\,dz - z_i e^{-z_i^2} \quad \text{and} \quad z_i = x/\sqrt{(4D_i t)};$$

and

$$\theta_r = \lambda S\left\{\frac{\alpha_i}{\sqrt{(4\pi D_i t)}}\, e^{-z_i^2}\right\}.$$

It follows that

$$\left(\frac{\partial r}{\partial \theta_r}\right)_t = -2x/\lambda.$$

Now

$$\begin{aligned}\bar{D}t &= S(\alpha_i D_i)\, t = \tfrac{1}{2}\overline{x^2} \\ &= \int_0^\infty \left(\frac{\lambda}{2}\frac{\partial r}{\partial \theta_r}\right)^2 \theta_r\,dx \Big/ \int_0^\infty \theta_r\,dx \\ &= \frac{\lambda^2}{24v}\int_0^\infty \theta_r . d\left\{\frac{\partial r}{\partial \theta_r}\right\}^3. \qquad (1)\end{aligned}$$

This quantity can be approximately computed from the Gouy interference pattern by the following procedure. The values of r for the interference minima, from without inwards are $\frac{3}{4}$, $1+\frac{3}{4}$, ...; the corresponding displacements of the minima from the optic axis, X_r are measured at a given time t. In addition, the maximal displacement $X_{\max.}$ is calculated from X_0/β_0 (Coulson *et al.* 1948; Ogston, 1949), which corresponds to $r=0$. r, X_r, δ_r, δX_r, $\dfrac{\delta r}{\delta X_r}$, $\left(\dfrac{\delta r}{\delta X_r}\right)^3$ and $\delta\left(\dfrac{\delta r}{\delta X_r}\right)^3$ are then tabulated. Since $\theta = X/F$, where F is the focal distance, equation (1) is approximated by

$$\bar{D}t = \frac{F^2\lambda^2}{24v} S\left\{X_r\,\delta\left(\frac{\delta r}{\delta X_r}\right)^3\right\}.$$

This sum is computed over the whole range of interference bands, including the optic axis where $X=0$ and $r=v$, but omitting the interference minimum next to the optic axis, since its proper value of r is uncertain (Kegeles & Gosting, 1947). By thus computing $\bar{D}t$ at two or more values of t, the value of $\bar{D}$ is obtained.

This method was tested on a record obtained with a nearly homogeneous sample of lactoglobulin. The mean diffusion

constant (in buffer at 20°) obtained from $\frac{1}{\sqrt{D}}=S\frac{\alpha}{\sqrt{D}}$ was $7{\cdot}15\times10^{-7}$, while the application of the above method gave $\bar{D}=6{\cdot}94$ and $7{\cdot}02\times10^{-7}$, from two intervals of time. The mean diffusion constant of a mixed solution of lactoglobulin and sucrose (Ogston, 1949) was found to be $10{\cdot}82\times10^{-7}$, the expected value being $10{\cdot}85\times10^{-7}$.

DISCUSSION

It is clear from the sedimentation diagrams that both polysaccharides are polydisperse, the dextrorotatory consisting of homogeneous and heterogeneous fractions and the laevorotatory being entirely heterogeneous. In addition, in neither case does all the refracting material appear in the sedimentation boundaries, which indicates that a proportion of the material may sediment too quickly or too slowly or may be too highly polydisperse to contribute to the boundary diagrams. Thus, while the mean sedimentation constants of the material can be regarded as established, in view of the uncertainties and errors discussed above neither the proportions of the components nor their diffusion constants should be regarded as more than rough estimates, and the same is true of the estimates of molecular weights, given in Table 2. These were derived from the sedimentation and diffusion constants assuming a value of 0·62 for the partial specific volumes.

The value of the mean diffusion constant of the laevorotatory polysaccharide, calculated by the method described, would give a reliable estimate of its mean molecular weight if it were certain that the average applied to the same range of material as does the estimate of the sedimentation constant. The fact that only 0·64 of the refracting material appears in the sedimentation boundary shows that this may not be so.

SUMMARY

1. Measurements of the sedimentation and diffusion of two samples of polysaccharide from *Penicillium luteum* are described.

2. A new method is given for analysing the diffusion data obtained with the Gouy diffusiometer, so as to obtain a value of the arithmetic-mean diffusion constant which is comparable with the mean sedimentation constant.

3. Approximate values for the amounts and constants of the components of the polysaccharides have been deduced.

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Metabolic Products of *Trichothecium roseum* Link

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The work described in this paper was carried out independently at Stevenston and the London School of Hygiene and Tropical Medicine. When the separate investigations were communicated to the Biochemical Society (Freeman & Morrison, 1948; Michael, 1948) the results from the two laboratories were found to be so similar that it was decided to present a joint account of the work. This paper deals with the isolation of three crystalline metabolic products of *Trichothecium roseum* which had not hitherto been described. Two of these products which are present mainly in the mycelium, and only in smaller amounts in the culture fluid, were named rosein I and rosein II, while the name rosein III is proposed for the third compound which has been found only in the culture fluid. The three new products are additional to trichothecin, the antifungal substance which was described by Freeman & Morrison (1949*a*).

It has been known for many years that fruits attacked by the pink rot caused by *T. roseum* Link contain a bitter principle. The latter was isolated in the form of a crude syrup and its solubility described by Iwanoff (1904). Antagonism between *T. roseum* and certain plant pathogenic fungi was reported by Whetzel (1909), Boning (1933), Koch (1934) and

Greaney & Machacek (1935). Koch (1934) stated that *T. roseum* actively parasitized the stroma of *Dibotryon morbosum* in the black knot disease of various hosts. Greaney & Machacek (1935) found that the fungus was antagonistic to *Helminthosporium sativum* when the two organisms were grown together in soil in the presence of cereal seedlings. The presence of an antifungal compound in culture filtrates of *Trichothecium roseum* was reported by Brian & Hemming (1947). The substance, trichothecin, responsible for the antifungal activity was isolated, and some of its chemical properties described by Freeman & Morrison (1949*a*). Its biological properties were described by Freeman & Morrison (1949*b*). Rosein I and crude rosein III were encountered as by-products in the isolation of trichothecin by Freeman & Morrison (1949*a*) and referred to in that paper as 'crystalline precipitate (II)' and 'precipitate (I)' respectively.

The mould was incubated for 3–4 weeks on Czapek-Dox medium which was enriched with corn-steep liquor. Extraction of the culture fluid with chloroform yielded a syrupy mixture from which rosein III was obtained as a colourless crystalline product by repeated precipitation with light petroleum from chloroform, followed by fractionation on an alumina column in chloroform-carbon tetrachloride. The petroleum mother liquors were evaporated and fractionated in ether by passing through an alumina column. The first fractions, on recrystallization from light petroleum containing some chloroform, yielded small amounts of roseins I and II; in the mother liquors of these products the bulk of the trichothecin was found.

Extraction of the dried and powdered mycelium with solvents yielded a mixture of two crystalline products, roseins I and II, the former in approximately double the amount of the latter. These products were accompanied in the extracts by considerable amounts of lipids.

In the London School of Hygiene and Tropical Medicine method, the extraction was carried out in a Soxhlet extractor with light petroleum. On concentrating and cooling the extract, a mixture of roseins I and II crystallized in yields of 2–3 % of the weight of mycelium. Separation of the two substances was effected by treatment with cold ethanol which leaves rosein I, m.p. 210°, undissolved, while rosein II, m.p. 186°, crystallizes from the mother liquors on addition of water.

In the Nobel Division method, boiling chloroform was used as solvent. Treatment of the concentrated extract with light petroleum afforded rosein I. On evaporation of the mother liquors and treatment with aqueous ethanol, rosein II was obtained.

Rosein I ($C_{19}H_{26}O_3$, m.p. 210°), which is slightly soluble in cold ethanol, crystallizes from this solvent in colourless tetrahedra. It is optically active, its specific rotation in chloroform being $[\alpha]_D$ −112·5°. It is a neutral compound of ketonic character, as shown by the formation of a 2:4-dinitrophenylhydrazone and an oxime. Under the influence of hot alkali, it is rearranged to a lactone, *iso*rosein I ($C_{19}H_{26}O_3$, m.p. 144°), which is no longer ketonic, but which possesses a hydroxyl group which can be benzoylated. Potassium permanganate at room temperature oxidizes rosein I to a monobasic acid, $C_{16}H_{24}O_4$, m.p. 242°.

Rosein II ($C_{19}H_{28}O_3$, m.p. 186°) crystallizes from aqueous ethanol or from toluene in colourless fibrous needles. It possesses weak optical activity, its specific rotation $[\alpha]_D$ in chloroform being +5·9°. It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. It is soluble in hot alkali and is recovered unchanged on acidification. Oxidation with potassium permanganate in acetone at room temperature gave a monobasic acid $C_{16}H_{26}O_5$. Rosein II was obtained from cultures on the enriched Czapek-Dox medium, but was not detected in cultures on Raulin-Thom medium.

Rosein III ($C_{20}H_{28}O_4$, m.p. 221°) crystallizes from toluene in colourless plates. It has a ketonic character as shown by the formation of a 2:4-dinitrophenylhydrazone. Hot alkali converts rosein III into an isomeric lactone, *iso*rosein III, $C_{20}H_{28}O_4$, m.p. 155°.

During the isolation of one of the strains used in this work (BB 105*a*) it was observed that an apple infected with the mould developed an intensely bitter taste, which permeated the whole of the fruit even when only a small area was infected. Filtrates from cultures of the mould on liquid media also possessed a bitter taste. An amorphous solid, similar in properties to Iwanoff's (1904) bitter principle, was isolated by ether extraction of the culture filtrate; trichothecin was shown to be one of its principal bitter components.

Antibiotic properties of the metabolic products. While roseins I and III were devoid of antibiotic activity against the test organisms, rosein II inhibited the growth of *Bacillus subtilis* at a concentration of 5 mg./l. It inhibited *Mycobacterium phlei* at 1:81,000, was only slightly active against *M. tuberculosis* and had no effect on *M. butyricum*.

EXPERIMENTAL

Organisms and cultural conditions

The organisms used for most of the work in Nobel Division were strains of *Trichothecium roseum* Link isolated from mouldy dead wood, laboratory catalogue numbers F 109 and F 227. They are described in detail by Freeman & Morrison (1949*b*). The strains used at the London School of Hygiene and Tropical Medicine were L.S.H.T.M., catalogue number BB 105*a*, isolated from an apple and identified by Mr G. Smith in 1945 and L.S.H.T.M., catalogue number 95, isolated from a tomato by Mrs Marcus in 1947.

The strains all grew well on Czapek-Dox agar and beer-wort agar and rapidly developed the typical pink conidial areas. Preliminary experiments showed that on liquid Czapek-Dox or Raulin-Thom media growth and sporulation were slow and sparse. Czapek-Dox solution (350 ml.) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 14 days, a thin mycelium had formed and pink conidial areas began to appear; at this stage the culture filtrate still contained approximately 4% glucose and its pH was 6·4. After incubation for 31 days the mycelium was thin, but well covered with spores. The pale yellow culture filtrate contained 3·2% glucose and its pH was 7·3.

After incubation for 7 days cultures on Raulin-Thom solution developed a white mycelium which had a tendency to sink below the surface of the liquid. Weak and patchy sporulation appeared after 24 days.

Czapek-Dox medium, which was enriched by the addition of 1% corn-steep liquor (50% total solids), sustained more rapid and abundant growth and spore formation. Czapek-Dox solution (350 ml.) + corn-steep liquor (3·5 ml.) in 1 l. conical flasks was inoculated with a heavy spore suspension and incubated at 24°. After 6 days a thick mycelium had formed and spore formation was beginning. After 14 days the mycelium was completely covered by pink conidial areas. The cultures were harvested after incubation for 21 days, when the pale yellow culture filtrate contained 0·7% glucose and its pH was 7·6.

The culture medium used in Nobel Division was based on the Czapek-Dox formula in which $NaNO_3$ was replaced by the equivalent quantity of ammonium tartrate and with the addition of 1% corn-steep liquor. The medium used at L.S.H.T.M. was Czapek-Dox solution with the addition of 1% corn-steep liquor.

Isolation of roseins I and II

Nobel Division method

Rosein I. The medium used contained ammonium tartrate (2 g.); $MgSO_4.7H_2O$ (0·5 g.); K_2HPO_4 (1·0 g.); KCl (0·5 g.); $FeSO_4.7H_2O$ (0·01 g.); crude commercial glucose (50 g.); corn-steep liquor (10 ml.) in water to 1 l. It was based on Czapek's formula (as modified by Dox, 1910), in which ammonium tartrate was substituted for $NaNO_3$ and corn-steep liquor was added as a supplementary nutrient. Crude commercial glucose ('glucose chips') was found to be a satisfactory source of sugar.

The medium (300 ml.) in 'Glaxo' bottles was sterilized by autoclaving. The reaction was adjusted to pH 5·0. The bottles were inoculated with a heavy spore suspension and incubated at 25° for 28 days in darkness.

The mycelium (strain F 109) was harvested and separated from the culture fluid by filtration through fluted filter paper, washed with cold water and dried in a current of air at 20°. Air-dried, powdered mycelium (159·2 g. from 15 l. of medium) was extracted three times under reflux for 0·5 hr. with boiling $CHCl_3$ (500 ml.). The combined extracts were concentrated, yielding an orange-yellow oily residue (36·3 g., 22·8% of mycelium). The residue was treated with light petroleum (b.p. 60–80°, 250 ml.) and a crystalline precipitate of crude rosein I (6·58 g., 4·13% of mycelium) slowly formed. The precipitate was collected, washed with light petroleum and extracted with cold ether. The mother liquor (*A*) and the ether extract (*B*) were retained. The crude rosein I was dissolved in $CHCl_3$ (5 ml.) and the solution passed through a column (20 × 1·5 cm.) of activated alumina in $CHCl_3$. The solvent was evaporated from the eluate and the residue (5·18 g.) was dissolved in the minimum volume of $CHCl_3$ (5 ml.) and diluted with ether (30 ml.). Slightly impure rosein I (m.p. 206°, 4·53 g.) crystallized out. The pure substance was obtained after one recrystallization from ethanol (4·11 g., 2·58% of mycelium, m.p. 210°) in the form of colourless tetrahedra.

Rosein II. The mother liquor (*A*), consisting mainly of a solution of lipids in light petroleum, was evaporated to dryness and the orange-coloured residue (29·71 g.) extracted for a few minutes with boiling 75% (v/v) aqueous ethanol (100 ml.). The extract was cooled, shaken with light petroleum (b.p. 60–80°, 30 ml.) and the ethanol layer separated and combined with the ether extract (*B*). On evaporation of

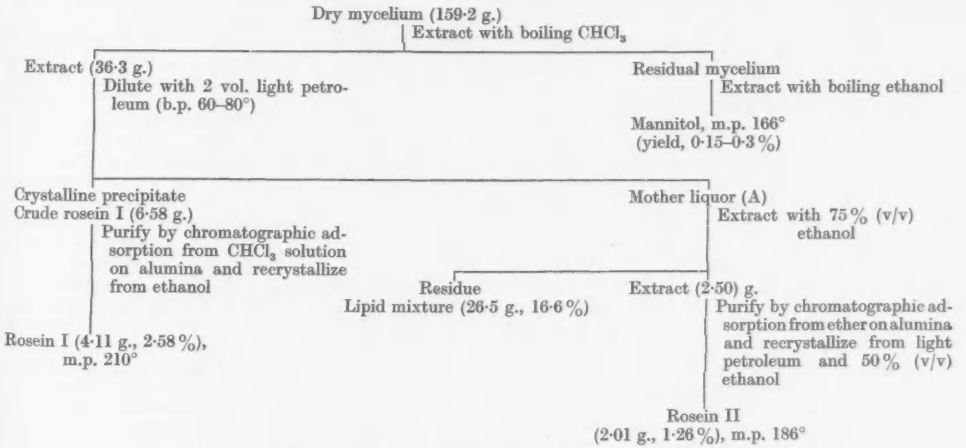
Dry mycelium (159·2 g.)
Extract with boiling CHCl3
Extract (36·3 g.)
Dilute with 2 vol. light petroleum (b.p. 60–80°)
Residual mycelium
Extract with boiling ethanol
Mannitol, m.p. 166°
(yield, 0·15–0·3%)
Crystalline precipitate
Crude rosein I (6·58 g.)
Purify by chromatographic adsorption from CHCl3 solution on alumina and recrystallize from ethanol
Mother liquor (A)
Extract with 75% (v/v) ethanol
Residue
Lipid mixture (26·5 g., 16·6%)
Extract (2·50) g.
Purify by chromatographic adsorption from ether on alumina and recrystallize from light petroleum and 50% (v/v) ethanol
Rosein I (4·11 g., 2·58%), m.p. 210°
Rosein II
(2·01 g., 1·26%), m.p. 186°

Fig. 1. Isolation of roseins I and II from mycelium.

the combined ethanol and ether solutions nearly to dryness crude rosein II crystallized (2·50 g.). It was dissolved in $CHCl_3$, boiled with a little activated charcoal and filtered. The residue, formed on evaporation of the filtrate, was recrystallized first from light petroleum (b.p. 60–80°) and then from 50% (v/v) aqueous ethanol giving pure rosein II (2·01 g., 1·26% of mycelium, m.p. 186°) in the form of colourless fibrous needles. Passage of an ether solution through a column of activated alumina was used in purification of some batches of crude rosein II.

The residue remaining after isolation of roseins I and II consisted mainly of a mixture of lipids, partially crystalline at 20°.

The fractionation of the mycelial products is summarized in Fig. 1.

London School of Hygiene and Tropical Medicine method

In several experiments 1 l. conical flasks (50), each containing Czapek-Dox solution (350 ml.) and corn-steep liquor (3·5 ml.) were inoculated and incubated for 21–26 days at 24°. At the end of this period the metabolism solution was separated from the mycelium by filtration and the latter well pressed, washed with water, dried *in vacuo* at 35° and finally ground to a fine powder. The yield from 50 flasks was 200–210 g. dry mycelium.

The culture filtrate was a clear, pale yellow liquid, pH 7·6. It contained 0·5–0·7% residual glucose. It gave no reaction with $FeCl_3$. The solution had an intensely bitter taste. After cooling to 5° for 24 hr., small amounts (0·1–0·2 g. from 17 l.) of colourless crystals had separated. They were filtered off, washed with water and dried *in vacuo*. The crystalline product consisted of a mixture of needles and tetrahedra identical with specimens of roseins I and II isolated from the mycelium.

The culture filtrate was three times extracted with ⅓ vol. of ether. The extract was filtered through a dry filter, evaporated to small volume, and then allowed to evaporate slowly. Small amounts (0·1–0·2 g.) of the same mixture of crystals separated. They were filtered off, washed with a little ether and dried *in vacuo*. The filtrate on evaporation to dryness gave an amorphous, buff-coloured powder (2 g.), having an intensely bitter taste. The product was slightly soluble in water, soluble in dilute NaOH giving a frothy solution and soluble in ethanol, acetone and ether.

Isolation and separation of metabolic products from mycelium. The dried, powdered mycelium was extracted in a Soxhlet extractor with light petroleum (b.p. 40–60°). After 3–4 hr. colourless crystals began to appear in the receiver. After 7 hr. the extract was yellow and had a strong smell of a fatty or oily character. At this stage the receiver was changed and extraction continued for four to five periods of 7 hr. The extracts were separately concentrated to small volume, kept at 0° for 16 hr. and the crystalline deposit filtered off, washed well with cold light petroleum and dried *in vacuo*. The product consisted of varying mixtures of colourless needles and tetrahedra. On evaporation of the combined mother liquors to small volume a further crop of crystals was obtained.

A typical extraction of dry mycelium (42 g.) gave from successive 7 hr. extractions 0·8 g. crystals, mainly needles; 0·3 g. crystals, a mixture; 0·1 g. crystals, mainly tetrahedra; and 0·05 g. crystals, mainly tetrahedra (total 1·25 g.; 2·98% of mycelium). The average yield, throughout the work, of the mixed crystalline product was 2–3% of the dry mycelium, equivalent to 0·3–0·4 g./l. of culture medium. The final mother liquors of the crystalline products, when freed from solvent, were semi-solid yellow oils.

The mixed crystalline product was separated into its components by extraction with cold ethanol in which rosein II was readily soluble, whilst rosein I was only slightly soluble. The mixture (2·85 g.) was suspended in ethanol (30 ml.) and stirred for 2 min. at room temperature. The colourless extract was filtered off and the insoluble residue washed with two 5 ml. portions of ethanol. The insoluble fraction was dried *in vacuo*; it consisted of rosein I, colourless tetrahedra (1·67 g., m.p. 210°).

The ethanolic mother liquor and washings of rosein I were combined, warmed to 60° and diluted with an equal volume of water at 60°. On cooling, colourless needles of rosein II separated. After standing at 0° for 16 hr., the product was filtered off, washed with ice-cold 50% (v/v) aqueous ethanol and dried *in vacuo*. Yield of rosein II, 0·8 g., m.p. 183°. The melting point of the product was raised to 186° on recrystallization from toluene.

The mother liquor of rosein II was freed from ethanol by evaporation and cooled to 0° for 16 hr. A deposit (0·3 g.) of a mixture of roseins I and II separated and was worked up with a subsequent batch. The initial crystalline mixture contained approximately two parts of rosein I and one part of rosein II.

Isolation of rosein III from culture filtrate

Rosein III was not obtained from the mycelium. It was extracted, together with trichothecin and small amounts of roseins I and II, by $CHCl_3$ from the culture filtrate (strain 227) and isolated by the following procedure.

The culture filtrate in lots of 1 l. was twice extracted with $CHCl_3$ (200 ml.). The combined extracts were evaporated to dryness under reduced pressure and the dried extract from 80 l. of culture filtrate was obtained as a brown gummy solid (21·23 g.). The latter was dissolved in $CHCl_3$ (20 ml.) and the solution diluted with ether (450 ml.). The resultant precipitate was rejected and the supernatant solution evaporated to dryness. The residue was redissolved in $CHCl_3$ (20 ml.) and the solution diluted with light petroleum (b.p. 60–80°, 450 ml.). The gummy precipitate which formed was separated by decantation, redissolved in $CHCl_3$ (10 ml.) and reprecipitated by addition of light petroleum as above. The extracts were combined and on evaporation to dryness gave 11·65 g. of an amorphous residue (*A*). The precipitate (*B*, 7·68 g.) was dissolved in 50% (v/v) $CCl_4/CHCl_3$ mixture and fractionated chromatographically on an alumina column (30 × 3·8 cm.) which was developed with a mixture of CCl_4 and $CHCl_3$. The eluate was collected in 50 ml. fractions, which after evaporation of the solvent were treated with a little ether. The crystalline fractions were combined (4·72 g.) and dissolved in a little $CHCl_3$. On addition of ether, rosein III crystallized out. The pure compound (3·68 g., m.p. 221°) was obtained after recrystallization from toluene as colourless plates.

The petroleum ether-soluble portion (*A*, 11·65 g.) containing trichothecin was dissolved in ether and fractionated chromatographically on an activated alumina column. The eluate was collected in 100 ml. fractions, the first three of which contained the bulk of the trichothecin. After evaporation of the solvent (ether) the fractions were dissolved in light petroleum (b.p. 60–80°) containing 5% (v/v) of $CHCl_3$. After standing at 5° for 16 hr., fraction II gave a crystalline deposit of rosein I (0·25 g.) and fraction III gave rosein II

(0·11 g.). After separation of these crystalline precipitates, the combined mother liquors were used for preparation of trichothecin. Fractions IV, V, VI and VII gave crystalline deposits of crude rosein III, totalling 2·11 g. The pure product (m.p. 221°) was obtained on recrystallization.

It was found to be most convenient and efficient to separate the fractions of (*A*) which were free from trichothecin (as determined by antifungal tests) and combine them with (*B*), for chromatographic purification of rosein III, as shown in Fig. 2. The total yield of rosein III was about 60 mg./l. of culture filtrate.

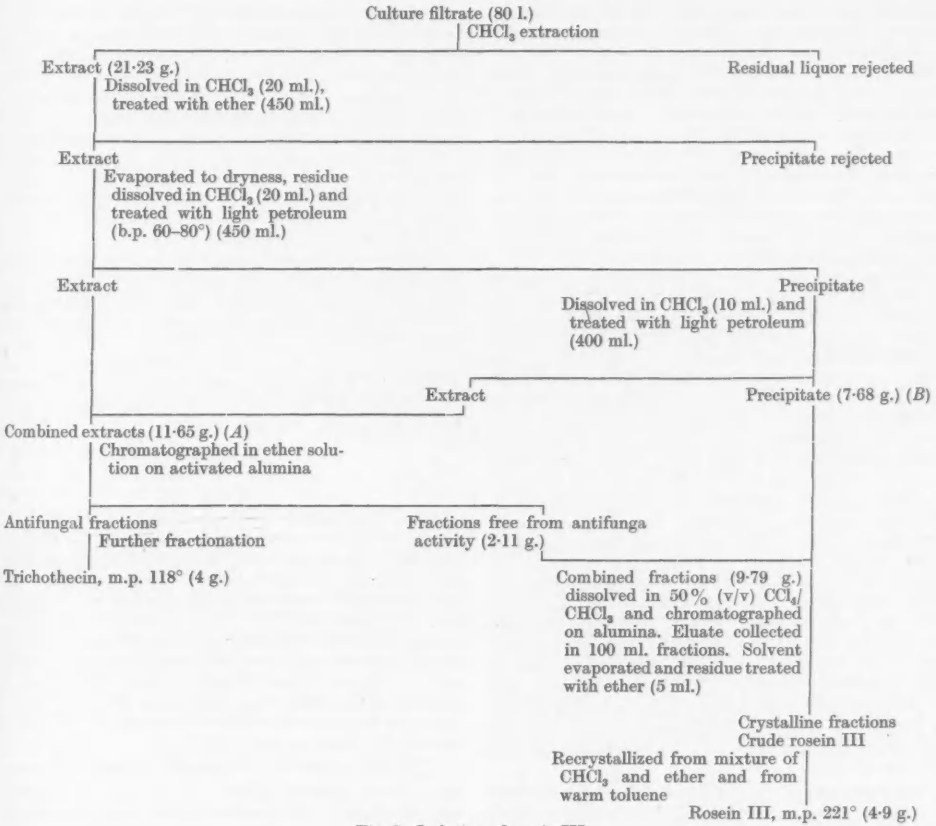
Culture filtrate (80 l.)
$CHCl_3$ extraction
Extract (21·23 g.)
Residual liquor rejected
Dissolved in $CHCl_3$ (20 ml.), treated with ether (450 ml.)
Extract
Precipitate rejected
Evaporated to dryness, residue dissolved in $CHCl_3$ (20 ml.) and treated with light petroleum (b.p. 60–80°) (450 ml.)
Extract
Precipitate
Dissolved in $CHCl_3$ (10 ml.) and treated with light petroleum (400 ml.)
Extract
Precipitate (7·68 g.) (*B*)
Combined extracts (11·65 g.) (*A*)
Chromatographed in ether solution on activated alumina
Antifungal fractions
Further fractionation
Fractions free from antifunga activity (2·11 g.)
Trichothecin, m.p. 118° (4 g.)
Combined fractions (9·79 g.) dissolved in 50% (v/v) CCl_4/$CHCl_3$ and chromatographed on alumina. Eluate collected in 100 ml. fractions. Solvent evaporated and residue treated with ether (5 ml.)
Crystalline fractions
Crude rosein III
Recrystallized from mixture of $CHCl_3$ and ether and from warm toluene
Rosein III, m.p. 221° (4·9 g.)

Fig. 2. Isolation of rosein III.

Properties and reactions of metabolic products

Rosein I

Rosein I crystallized from hot ethanol in colourless tetrahedra, m.p. 210°. It was readily soluble in $CHCl_3$ or acetone and was fairly soluble in benzene, slightly soluble in ether or ethanol, very slightly soluble in cold light petroleum and practically insoluble in water, cold dilute NaOH and dilute HCl. It was optically active: $[\alpha]_D^{24°} - 112·5°$, $[\alpha]_{5461}^{24°} - 140°$ in $CHCl_3$ (*c*, 1). Nitrogen, halogens and sulphur were absent. (Found: (Weiler and Strauss): C, 75·4, 75·4; H, 8·9, 8·8; mol. wt. (cryoscopic in benzene), 302; (Rast), 316; methoxyl, nil; active hydrogen, nil. $C_{19}H_{26}O_3$ requires C, 75·5; H, 8·6%; mol. wt. 302.)

Rosein I dissolved in conc. H_2SO_4 to give a pale orange-yellow solution. The compound gave no colour reactions for sterols. A solution in aqueous ethanol was neutral, gave no reaction with $FeCl_3$ and did not reduce Fehling solution or Tollens ammoniacal $AgNO_3$ reagent. There was no reaction with Br_2 in CCl_4. An acetone solution of $KMnO_4$ was slowly decolorized at room temperature. Alkaline $KMnO_4$ was not decolorized at room temperature, but was rapidly decolorized on warming. Rosein I gave no benzoyl derivative on treatment with benzoyl chloride and pyridine.

Rosein I 2:4-*dinitrophenylhydrazone.* Rosein I (100 mg.) was dissolved in glacial acetic acid (5 ml.) and mixed with 2:4-dinitrophenylhydrazine (150 mg.) in glacial acetic acid (10 ml.) After 18 hr., the mixture was diluted with 2N-HCl and the crystalline precipitate filtered off and washed first with 2N-HCl and then with water until neutral. The crude

product (147 mg.) was dissolved in CCl_4 and purified chromatographically on activated alumina. The main band and several minor bands were developed and eluted by gradually increasing the proportion of $CHCl_3$ in the solvent. The main fraction (120 mg.) crystallized from aqueous ethanol in the form of yellow needles, m.p. 239°.

The same product was obtained by treatment of rosein I (100 mg.) in absolute ethanol with 1·5 ml. Brady reagent (1 g. 2:4-dinitrophenylhydrazine in 2 ml. conc. H_2SO_4 and 15 ml. ethanol). Yellow needles began to crystallize after 30 min., and the product was collected after 20 hr. at room temperature. The hydrazone in ethanolic solution gave an intense red colour with strong alkali. (Found: C, 62·7, 62·3; H, 6·3, 6·3; N, 11·3, 12·5. $C_{25}H_{30}O_6N_4$ requires C, 62·2; H, 6·2; N, 11·6%.)

Rosein I oxime. Rosein I (100 mg.) dissolved in warm ethanol (5 ml.) was treated with hydroxylamine hydrochloride (30 mg.) and anhydrous sodium acetate (200 mg.). The mixture was kept at 60° for 1 hr. and most of the ethanol distilled off. The residue was diluted with water and extracted with ether. The extract was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue (96 mg.) was recrystallized from aqueous ethanol and formed colourless prisms, m.p. 215°. (Found: C, 71·7; H, 8·5; N, 4·3, 4·5. $C_{19}H_{27}O_3N$ requires C, 71·9; H, 8·5; N, 4·4%.)

iso*Rosein I.* Rosein I (220·8 mg.) was heated under reflux for 6 hr. with 0·1 N-ethanolic KOH (20 ml.). The solution was cooled, diluted with water and titrated with 0·1 N-HCl. (Found: 220·8 mg. rosein I equiv. to 7·56 ml. 0·1 N-HCl; saponification value, 292 (theory, 302).) The bulk of the ethanol was distilled off and the residue made acid to congo red indicator by addition of conc. HCl. The resultant colourless precipitate was extracted with ether. The extract crystallized slowly on evaporation of the solvent giving colourless crystalline *iso*rosein I (190 mg., 86·1% yield). The product was recrystallized from light petroleum (b.p. 60–80°) and then from 50% (v/v) aqueous ethanol in the form of colourless prisms, m.p. 144°. *iso*Rosein I was readily soluble in cold ethanol, ether, $CHCl_3$, acetone and benzene, slightly soluble in light petroleum and insoluble in water. It dissolved slowly in hot 2 N-NaOH. No precipitate appeared on cooling. On addition to the cold alkaline solution of an equal volume of phosphate buffer, pH 6, no precipitate appeared. On acidifying with 2 N-HCl, *iso*rosein I, m.p. 143°, crystallized. It did not dissolve in $NaHCO_3$ solution and had no acidic properties. (Found: C, 75·7, 75·4; H, 8·5, 8·9; mol. wt. (cryoscopic in benzene), 280. $C_{19}H_{26}O_3$ requires C, 75·5; H, 8·6%; mol. wt. 302.) $[\alpha]_{5461}^{20°}$ +40° (*c*, 1 in ethanol). *iso*Rosein I did not react with hydroxylamine or with 2:4-dinitrophenylhydrazine.

Monobenzoyl iso*rosein I.* *iso*Rosein I (200 mg.), dissolved in pyridine (5 ml.), was treated with benzoyl chloride (500 mg.). After standing at 20° for several days, the mixture was poured into ice-cold dilute HCl and extracted with ether. The extract was washed first with water, then with dilute NaOH and finally with water until neutral. On evaporation of the solvent, the residue crystallized slowly. It was decolorized by solution in ether and passage through a column of activated alumina. Recrystallization from aqueous ethanol gave colourless needles of monobenzoyl *iso*rosein I, m.p. 156° (180 mg.). (Found: C, 75·7; H, 7·6. $C_{26}H_{30}O_4$ requires C, 76·9; H, 7·4%.)

Hydrolysis of monobenzoyl iso*rosein I.* Monobenzoyl *iso*-osein I (81 mg.) was dissolved in ethanol (10 ml.) and heated under reflux with 0·1 N-KOH (10 ml.). Titration with 0·1 N-HCl showed that roughly 2 equiv. of alkali had been taken up, which was in accordance with the hypothesis that 1 equiv. was taken up in hydrolysis of the benzoate and 1 equiv. in hydrolysis of a lactone ring. The solution was made acid to congo red and allowed to stand for 24 hr. A slight excess of $NaHCO_3$ was then added and the solution extracted with ether. The extract was washed with water, dried over Na_2SO_4 and the solvent evaporated off. The crystalline residue was recrystallized from ethanol giving colourless crystals, m.p. 213–214°, in insufficient quantity for analysis.

Permanganate oxidation of rosein I. Rosein I (50 mg.) was dissolved in a 1% solution of $KMnO_4$ in acetone (5 ml.). After 1 hr. the purple colour had disappeared and MnO_2 had been precipitated. A further 5 ml. of $KMnO_4$ solution was added; after standing for 16 hr. the purple colour had again been discharged. The MnO_2 was filtered off, washed well with acetone and dried. On evaporating the filtrate and washings, rosein I (37 mg.) was recovered. The MnO_2 was twice extracted with 10 ml. portions of boiling water. On acidification of the colourless extract with HCl, sheaves of colourless needles soon crystallized out. They were collected after 16 hr. at 0°, m.p. 242° (7·5 mg.).

In a second experiment, rosein I (50 mg.) was treated with 1% $KMnO_4$ in acetone (25 ml.). After 3 days at room temperature, the permanganate was nearly decolorized and the solution was decolorized by passage of SO_2. The mixture was worked up as described above and the same product isolated (8·5 mg.). (Found on product dried to constant weight *in vacuo* at 60°: C, 68·2; H, 8·1; acid equiv.: 5·4 mg. required 0·18 ml. 0·1 N-NaOH (cold or hot) giving equiv. for one acidic group, 300. $C_{16}H_{24}O_4$ requires C, 68·6; H, 8·6%; equiv. 280.)

Rosein II

Rosein II crystallized from light petroleum (b.p. 60–80°), toluene or 50% (v/v) aqueous ethanol in long fibrous needles, m.p. 186°. It was practically insoluble in water and cold 2 N-NaOH, only slightly soluble in light petroleum, but readily soluble in most other organic solvents, such as ether, acetone, ethanol and $CHCl_3$. Its solution in conc. H_2SO_4 was deep orange yellow. There was no reaction with ethanolic $FeCl_3$. The Salkowski and Liebermann-Burchard reactions for sterols were negative. A solution of rosein II in aqueous ethanol was neutral and had no reducing properties. A 2:4-dinitrophenylhydrazone could not be prepared. Rosein II was weakly dextrorotatory; $[\alpha]_D^{23°}$ +5·9° in $CHCl_3$ (*c*, 2); $[\alpha]_{5461}^{20°}$ +7·5° in ethanol (*c*, 1). Nitrogen, sulphur and halogens were absent. (Found: C, 75·2, 75·3, 75·4; H, 9·3, 9·5, 9·3; mol. wt. (Rast) 278, 337; methoxyl, nil; active hydrogen, nil. $C_{19}H_{28}O_3$ requires C, 75·0; H, 9·2%; mol. wt., 304.)

Action of alkali. Rosein II (0·15 g.) was dissolved in ethanol (50 ml.) and 10 N-NaOH (3 ml.) added. The clear solution was heated under reflux on a steam bath for 2 hr. After cooling and removing about half the ethanol *in vacuo*, the solution was diluted with water (30 ml.) and acidified with 2 N-HCl; rosein II (0·135 g., m.p. 186°) crystallized as colourless needles.

Permanganate oxidation. Rosein II (1·008 g.) was dissolved in a 1% solution of $KMnO_4$ in acetone (200 ml.). After 16 hr. at 25° the purple colour had almost disappeared. The precipitated MnO_2 was filtered off, washed well with

acetone and dried. On evaporating the acetone filtrate and washings unchanged rosein II (0·3157 g., m.p. 186°) was recovered.

The dried MnO_2 was twice extracted with boiling water (200 ml.). On cooling there was a small deposit (7·5 mg.) from the first aqueous extract. The filtrate was acidified by addition of conc. HCl (2·5 ml.) giving an immediate amorphous colourless precipitate of oxidation product (0·2672 g.). The latter was crystallized from 50% (v/v) aqueous ethanol (17 ml.) yielding colourless hexagonal plates (0·098 g., m.p. 296°). (Found: C, 64·1, 64·2; H, 8·5, 8·7. $C_{16}H_{28}O_5$ requires C, 64·5; H, 8·8%.) Acid equiv.: 32·6 mg. required 2·14 ml. 0·05 N-NaOH (hot or cold) in aqueous ethanolic solution giving equiv. for one acidic group, 305 (theory 298).

Rosein III

Rosein III crystallized from toluene in colourless plates, m.p. 221°. It was only very slightly soluble in water or light petroleum (b.p. 60–80°), slightly soluble in ether and readily soluble in $CHCl_3$, or ethanol. $[\alpha]_D^{19°} - 124°$ in $CHCl_3$ (*c*, 1). Nitrogen, sulphur and halogens were absent. (Found: C, 72·1, 72·5, 72·5; H, 8·3, 8·5, 8·5; methoxyl, nil; mol. wt. (Rast) 339. $C_{20}H_{28}O_4$ requires C, 72·3; H, 8·4; mol. wt., 332.)

In aqueous ethanolic solution, rosein III was neutral, had no reducing properties and gave no reaction with $FeCl_3$. On treatment with acetic anhydride in glacial acetic acid, the compound (m.p. 221°) was recovered unchanged.

Rosein III 2:4-*dinitrophenylhydrazone.* Rosein III (0·110 g.) in ethanol (2 ml.) was treated with Brady reagent (1·5 ml.) as described above. The crude hydrazone (0·167 g.) was purified chromatographically from ether solution on activated alumina. The main fraction was crystallized from ethanol as yellow fibrous needles (0·107 g., m.p. 280° decomp.). (Found: C, 60·2; H, 6·2; N, 11·3. $C_{26}H_{32}O_7N_4$ requires C, 60·8; H, 6·2; N, 11·0%.)

iso*Rosein III.* Rosein III (0·5 g.) dissolved in 6% methanolic KOH (25 ml.) was heated under reflux for 4 hr. The solution was cooled, diluted with water and the methanol distilled off *in vacuo*. The residue was acidified with HCl and extracted with $CHCl_3$. The extract was washed, dried over Na_2SO_4 and evaporated almost to dryness. The residue was a pale yellow syrup which crystallized slowly. The crude product was recrystallized from toluene and obtained as colourless microcrystalline needles (0·135 g., m.p. 155°). (Found: C, 71·9; H, 8·3; mol. wt. (Rast) 272. $C_{20}H_{28}O_4$ requires C, 72·3; H, 8·4%; mol. wt., 332.)

Biological properties of metabolic products

Roseins I, II and III were tested for antibiotic activity against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Penicillium digitatum*. The metabolic products in 0·1% solution in ethanol were placed in porcelain cylinders embedded in suitable media in Petri dishes, the media having been seeded with one of the test organisms. The plates were examined for zones of inhibition after 24 hr. incubation at 32° in the case of the bacterial cultures and incubation for 5 days at 25° for *P. digitatum*. There was no inhibition of growth except that of *B. subtilis* by rosein II, which gave a zone of partial inhibition of 2·5 cm. diameter. In serial dilution tests *B. subtilis* grew normally in nutrient broth medium containing 2·5 mg. rosein II/l., but there was complete inhibition at 5·0 mg./l.

Weak antibacterial activity against *B. subtilis* was observed in filtrates of cultures of *T. roseum* F 109, grown on Czapek-Dox medium, by the cylinder-plate technique at an early stage in the work. This is now shown to be due to the presence of rosein II. Dilutions of the culture filtrates were only feebly active since the solubility of rosein II in water at 20° does not greatly exceed its limiting antibacterial concentration.

The effect of rosein II on the growth of three species of acid-fast bacteria was investigated by Dr A. R. Martin. The test against *Myco. tuberculosis* was carried out by incorporating the substance in an agar medium and inoculating the organism on to the surface. After incubation for 14 days the organism grew poorly in medium containing a concentration of 1:3000 of rosein II and some inhibition was still apparent at a concentration of 1:81,000. *Myco. phlei* and *Myco. butyricum* were grown on liquid media in which rosein II had been dispersed and the observations made after incubation for 2 days. Growth of the former was completely inhibited at 1 in 81,000, but normal growth took place at 1 in 243,000. Normal growth of the latter took place in the presence of 1 part rosein II in 1000.

Isolation of trichothecin as a component of the bitter principle of Trichothecium roseum

Iwanoff (1904) first reported that the metabolic products of *T. roseum* contained a very bitter substance. This was also noticed by the present authors and by Yasue (1948, private communication), but the bitter components have not hitherto been characterized.

The culture filtrate from 50 cultures of strain BB 105*a* (17·5 l.) was three times extracted with 2 l. lots of ether. On evaporation, an amorphous, buff-coloured powder (2 g.) of intensely bitter taste was obtained. A sample (36·2 mg.) was dissolved in ethanol (1 ml.) and diluted to 100 ml. with water giving an opalescent solution. After suitable dilution, the solution was assayed for antifungal activity by the *Penicillium digitatum* spore-germination technique which showed that a powerful antifungal compound was present. The ether extract (1·48 g.) was fractionated by the method described by Freeman & Morrison (1949*a*) for the isolation of trichothecin. The combined light petroleum (b.p. 60–80°) extracts (0·80 g.) were dissolved in ether (10 ml.) and fractionated on a column of activated alumina (25 × 2·0 cm.). The chromatogram was developed with ether and the eluate collected in 100 ml. fractions. These were evaporated to dryness and treated with light petroleum containing 5% (v/v) $CHCl_3$ (5 ml.). Fraction 2 gave crystalline trichothecin (57·5 mg.), m.p. and mixed m.p. 118°. Fractions 5 + 6 gave crystalline rosein II (14·2 mg.), m.p. 186°.

The fraction insoluble in light petroleum was fractionated as described in the preparation of rosein III, but only traces of uncharacterized crystalline fractions were obtained. No rosein III was detected.

Tasting tests by four independent observers showed that the water-insoluble compounds, roseins I and II, were tasteless; rosein III was slightly bitter, and trichothecin and *iso*trichothecin (hitherto referred to as the alkaline hydrolysis product of trichothecin by Freeman & Morrison, 1949*a*) were very bitter.

The available evidence suggests that trichothecin is responsible for part, at least, of the bitter taste of *T. roseum* culture filtrates and the bitterness of fruits attacked by the fungus.

Carbon balance of metabolic products of Trichothecium roseum

In order to determine whether trichothecin and roseins I, II and III, isolated in yields of 50, 3, 1 and 61 mg./l. respectively from modified Czapek-Dox medium, represented the main soluble metabolic products of *T. roseum*, partial carbon balance sheets of four strains grown on Czapek-Dox medium were prepared. The results (Table 1) showed that of the carbon in the initial glucose, 7–15% remained in the culture filtrate in the form of non-volatile components other than glucose and 11–18% was found in the mycelium. There was no production of acidic substances as shown by a steady rise of pH during incubation, until a final value of pH 7–9 was reached. The observations were based on the bulked filtrates from two 'Glaxo' bottle cultures (350 ml.), of each strain, which were diluted to 1 l. for analysis; initial pH 6·6. Portions (100 ml.) of the diluted filtrates were twice extracted with $CHCl_3$ (50 ml.), the aqueous fraction freed from $CHCl_3$ by evaporation and carbon determined by the wet combustion method of Houghton (1945). The carbon extracted from the culture filtrate by $CHCl_3$ represented less than half of the non-volatile carbon of the metabolic products. The carbon accounted for by the four known metabolic products, i.e. 81 mg./l. of the culture filtrate, was small compared with the total $CHCl_3$-soluble carbon of the filtrate (mean for the four strains, 792 mg./l.) and it is clear that the greater part of the soluble metabolic products of the fungus remain to be identified.

The carbon balance of a culture of *T. roseum* (*Cephalothecium roseum* Corda) was determined by Birkinshaw, Charles, Hetherington & Raistrick (1931). It was shown that 'this species grew well on Czapek-Dox solution, but was only able to metabolize very slowly the glucose supplied, since, even after 67 days, only about 65% of the glucose had been utilized'. The fungus was shown to have a respiration coefficient of 1·19.

Table 1. *Partial carbon balance of metabolic products of four strains of* Trichothecium roseum

Strain no.	Initial glucose carbon (g./100 ml.)	Period incubation (days)	Residual glucose carbon (g./100 ml.)	Total carbon in solution (g./100 ml.)	Carbon in volatile neutral compounds (g./100 ml.)	Carbon in solution 'unaccounted for' by difference (as % of original glucose carbon)	Carbon extracted from culture filtrate by $CHCl_3$ (g./100 ml.)	$CHCl_3$-extracted carbon (% of original glucose carbon)	Mycelium dry weight (g.)	Carbon content of mycelium (%)	Mycelial carbon (% original carbon)	Fungistatic activity as trichothecin after incubation for 42 days (mg./l.)	Final pH at time of harvest
109	1·317	58	0·012	0·169	0·009	11·24	0·070	5·31	4·81	49·89	18·2	4·5	7·2
198	1·317	71	0·044	0·194	0·006	10·93	0·029	2·20	3·87	53·77	15·8	27·1	7·1
227	1·317	42	0·056	0·153	0·006	6·90	0·022	1·67	4·38	51·17	17·0	28·9	7·8
271	1·317	42	0·024	0·178	0·002	14·53	0·088	6·68	3·14	48·54	11·6	18·7	8·9

Properties of the lipids

The $CHCl_3$ extract of the mycelium consisted mainly of a liquid mixture of esters, which amounted to 20% of the weight of the dry mycelium. The crude oil, dark brown in colour, was decolorized by passage through a column of charcoal. The almost colourless filtrate was liquid at 20° and crystallized partially below 15°. It had the following constants: saponification value, 194; iodine value, 57.

After saponification, the fatty acids were separated into saturated and unsaturated fractions by the lead salt method (Twitchell, 1921). The mixed fatty acids (151 g.) yielded 47 g. (31%) of saturated and 95 g. (63%) of unsaturated acids. The latter contained a small proportion of linoleic acid, but no linolenic acid was detected. The major component was probably oleic acid. A portion of the unsaturated acids (0·5 g.) was dissolved in $CHCl_3$ and excess bromine added. When the reaction was complete, the excess bromine and $CHCl_3$ were distilled off. The residue was completely soluble in ether indicating the absence of linolenic acid. After distillation of the ether, the residue was dissolved in hot light petroleum (b.p. 60–80°). On cooling colourless needles of linoleic acid tetrabromide (0·05 g.) separated. Recrystallization from light petroleum gave soft needles, m.p. 115° (literature value, m.p. 114–115°).

SUMMARY

1. The isolation and properties of roseins I and II from the mycelium of *Trichothecium roseum* and of rosein III from the culture filtrate are described.

2. Rosein I, colourless tetrahedra, m.p. 210°, $[\alpha]_D^{24°} - 112{\cdot}5$ in chloroform (*c*, 1) has the molecular formula $C_{19}H_{26}O_3$. It is a neutral ketone, which reacted with one equivalent of hot ethanolic alkali and yielded a lactone, *iso*rosein I, m.p. 144°, on acidification. The latter has an hydroxyl but no ketonic group. On oxidation with potassium permanganate, rosein I gave a monobasic acid, $C_{16}H_{24}O_4$, m.p. 242°.

3. Rosein II, $C_{19}H_{28}O_3$, crystallized in colourless fibrous needles, m.p. 186°, $[\alpha]_D^{23°} + 5{\cdot}9°$ in chloroform (*c*, 2). It has no ketonic or hydroxyl groups, but its properties correspond to those of a lactone. Oxidation with potassium permanganate gave a monobasic acid, $C_{16}H_{26}O_5$, m.p. 296°.

4. Rosein III, $C_{20}H_{28}O_4$, crystallized from toluene in colourless plates, m.p. 221°, $[\alpha]_D^{19°} - 124°$ in chloroform (*c*, 1). It was a neutral ketone which, on treatment with hot alkali, yielded *iso*rosein III, m.p. 155°.

5. Rosein II inhibited growth of *Bacillus subtilis* at 5 p.p.m. It was slightly active against *Mycobacterium tuberculosis*, inhibited *Myco. phlei* at 1 in 81,000 and had no effect on *Myco. butyricum* at 1 in 1000. Roseins I and III had no effect on three test bacteria and on *Penicillium digitatum*.

6. The bitter principle of *Trichothecium roseum* observed by Iwanoff (1904), and others has been shown to contain trichothecin.

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Note added in proof. A paper has recently appeared by Robertson, A., Smithies, W. R. & Tittensor, E. (*J. chem. Soc.* 1949, p. 879) describing the isolation of rosenonolactone and rosonolactone from *Trichothecium roseum* Link. It appears probable that these two substances are identical with roseins I and II respectively.

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Acid-soluble Pigments of Molluscan Shells

2. PIGMENTS OTHER THAN PORPHYRINS

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The object of the study described in this paper was to identify, if possible, the acid-soluble shell pigments which accompany shell porphyrins in several groups of marine molluscs (Comfort, 1948, 1949*a*) or which occur in related genera, and to examine their chromatographic behaviour and phylogenetic distribution. Work was concentrated on the shell pigments of the bivalve genus *Pinctada*, in the hope of relating them to the metabolism of porphyrins. This paper contains a summary of their chromatographic behaviour and general properties, compared with those of other acid-soluble molluscan shell pigments.

Previous work on shell pigments contains descriptions of a number of substances, but no clear picture of their chemical and phylogenetic relationships. Linear tetrapyrroles are known to occur in shells (Krukenberg, 1883; Schultz, 1904). Tixier (1947) isolated a number of fractions by chromatography of acid methanol extracts of *Turbo regenfussi* and *T. marmoratus*, and identified the main crystallizable shell pigment as a glaucobilin (Turboglaucobilin). Dhéré & Baumeler (1929) regarded the red pigment of *Haliotis refuscens* as a pyrrole derivative. The blue pigment of *H. californiensis* has been variously described as an indigoid

and as a tetrapyrrole (Krukenberg, 1883; Schultz, 1904, 1931; Kodzuka, 1921; Schultz & Becker, 1931; Lemberg, 1931); its investigation will form the subject of a separate paper. The pigments described below appear to be distinct both from the known indigoids and from the bile pigments.

EXPERIMENTAL

Extraction. Coarsely broken shell was macerated for 12–24 hr. at room temperature with conc. HCl, or with 33% (v/v) aqueous H_3PO_4 (sp.gr. 1·75 diluted 1 in 3). Octanol was added to prevent frothing. When extraction was complete, the coloured solutions were filtered through cellulose wool, and the pigments separated by chromatography.

Chromatography. This was carried out directly from aqueous acid solution, with talc as the adsorbent. Talc of normal pharmaceutical quality was shaken with 3 N-HCl and poured through a funnel containing glass beads into tubes, the lower ends of which were plugged with cotton wool supported on hollow ceramic insulators. The columns were then packed by suction, with frequent tapping to expel bubbles.

The solutions for chromatography were adjusted by dilution to an acidity of N- to 2·5 N-HCl, and passed through the columns under suction. Under these conditions the porphyrins tend to form a sharp zone, detectable by ultraviolet fluorescence, close to the top of the column. The concomitant pigments falling below this zone were eluted with increasing strengths of acetone in 2 N-HCl or H_3PO_4.

The adsorptive power of different samples of talc varied, and results given in this paper refer to a single batch of known behaviour. Increase in the acidity of the solvent above 2 N-HCl produced blurring of the zones, and, with some extracts, reversal of the order of adsorption.

Spectroscopy. Visible absorption bands were measured with a Hartridge reversion spectroscope. All wave lengths are stated in mμ.

RESULTS

Distribution

The distribution of non-porphyrin acid-soluble shell pigments closely follows that of the porphyrins. In general, the pigments of higher molluscs cannot be separated from the conchiolin of the shell. They appear to be chromoproteins. Absorption spectra obtained by trans-illuminating the shell give no clue to the nature of the chromophore.

Acid-soluble pigments occur in, and account for the colour of, most archaeogastropod shells, the pearl mussels, the purple Anomiidae such as *Placuna* and the tectibranch opisthobranchs. In the archaeogastropods their range is wider than that of the shell porphyrins, since they occur in the porphyrin-free species of Trochidae, in the Turbinidae and in *Haliotis*. The red pigment of recent *Pleurotomaria*, which could not be obtained for extraction on account of their rarity, is probably also acid soluble.

Comparison of chromatograms

The chromatograms of a number of pearl-mussel shell extracts show a close general similarity. In several species the lowest major pigment band was pink, the second blue and the third violet. In these extracts the porphyrin fraction, varying in colour from violet to brown, appeared higher on the column than the non-porphyrin pigments.

Preliminary study of the chromatograms of extracts of archaeogastropod shells shows a greater diversity of patterns. Chromatograms of species of *Fissurella*, *Trochus*, and *Clanculus*, contained pigment bands similar in appearance and sequence to those of the bivalves, but lack of material prevented further study of these forms.

In all forms, quantitative variation in the bands was marked, and the chromatograms of pooled extracts differed from those of single specimens. This variation was particularly marked in *Pinctada vulgaris*.

In the following protocols, typical chromatograms of several forms are described in detail. The species of *Haliotis* and *Turbo* are included for comparison with the bivalves, since they contain pigments which have been studied by other workers.

CHROMATOGRAPHY OF SHELL EXTRACTS

Pinctada vulgaris

Six valves were broken and macerated overnight in a mixture of 170 ml. H_3PO_4 (sp.gr. 1·75) and 430 ml. water. The port-wine coloured extract was filtered and chromatographed on a column of talc, 20 × 3 cm. The undeveloped chromatogram showed the following bands (Fig. 1):

Band no.	(Top)	Width (mm.)	
	Yellow	3	
	Grey	3	
	Ochre	3	
5	Brown	6	red fluorescence
4	Pink	2	red fluorescence
3	Blue	6	
2	Pink	15	
	Blank	6	
1	Purple	3 moving down	

Band 1 was eluted by further addition of solvent. Bands 2 and 4 were removed by approx. 3% (v/v) acetone in 33% aqueous H_3PO_4 as a single pink, non-fluorescent fraction (absorption bands 545, 497 mμ.). Band 3 moving down divided into two, a lower purple and an upper indigo blue (3*a*), both showing pink fluorescence. The fraction containing these bands gave absorption maxima at 593, 495, 465 mμ. in N-HCl. The brown band containing most of the porphyrin, accompanied by a non-fluorescent brown band, and another non-fluorescent purple band, not separable on the column, was eluted with 30% (v/v) acetone in acid, the mixed eluate giving absorption bands at 593, 572, 550 (intense), 491·5 (faint), 467 mμ.

Rechromatography of porphyrin fractions. The porphyrin fraction was warmed and the acetone removed by a current

of air. The solution was passed through a talc column 10 × 0·75 cm., giving four zones, from above: yellow, pink (strong fluorescence), yellow, blue-green. The two lower bands were removed by development with 1% pyridine in 33% H_3PO_4, and the remainder by 10% (v/v) aqueous pyridine. The second eluate was diluted with twice its volume of water and again chromatographed, giving a wide pink zone. Traces of green pigment passed out in the solvent. The column was developed with aqueous acetone (1 part in 6 parts of water).

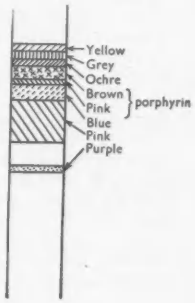

Fig. 1. Chromatogram on talc of acid extract of *Pinctada vulgaris* shells (crude extract; see p. 200).

A wide porphyrin band passed down, leaving another, equally fluorescent but pinker, to be removed by 90% aqueous acetone. The spectra of these fractions differed (Table 1): the spectra became identical on addition of excess 3N-HCl: upper band—624, (606·5), 590–568, 535, 490 mμ.; lower band—567, 541, 504 mμ. On addition of conc. HCl to both fractions, the spectra became identical: 594, 574, 550 mμ. Both solutions were clear violet in colour.

Rechromatography of non-porphyrin fractions. Fraction 1 was adsorbed on a small (10 × 1 cm.) column, and eluted with acetone as a brownish purple solution, in which no band could be detected visually.

Fraction 2, though not fully homogenous on readsorption, could not be further resolved on talc. Its spectral absorption curve is shown in Fig. 2.

Fraction 3–3*a*, which was manifestly complex on the first column, was diluted and rechromatographed on a 10 × 1 cm. column. It gave a greyish upper zone, a purple band and a narrow indigo-blue zone, and on further development with acid acetone (approx. 1 part acetone + 9 parts of 20% (v/v) aqueous H_3PO_4), the purple band could be seen to overlie a wider blue zone which overlapped it on each side, but travelled down with it. The purple band had now a very strong red fluorescence of typical porphyrin appearance, but this was evident only in adsorbed material; the eluate, a clear-cut, violet fraction, was non-fluorescent both on isolation and after addition of 0·25 ml. conc. HCl to about 3 ml. of eluate. It showed strong bands at 507–498 mμ. max. 500 mμ.; 608–578 mμ. max. 602 mμ.

The column was now washed down with 50% acetone in 20% (v/v) H_3PO_4, and a purple-brown band was recovered. It showed a single faint adsorption band about 500 mμ.

Subsequent extraction and working up of large amounts of material, especially re-extracts and dregs of spent shell, gave larger proportions of the 3–3*a* fraction, having a strong fluorescence both on the column and in solution. Spectroscopy of these fractions in the ultraviolet showed no Soret

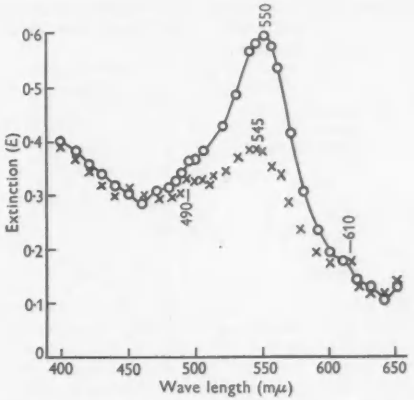

Fig. 2. Spectral absorption (in N-HCl-acetone) of lower pigment fractions from chromatograms of shell extracts (Beckmann spectrophotometer): o—o—o, *Pinctada vulgaris*; ×—×—×, *Malleus regula*.

band at a cell depth of 0·25 cm., but a trace ($\lambda = 405$ mμ.) at 1 cm., insufficient to account for the colour of the fraction. The inequality of these results and the lack of relation between colour and fluorescence in successive specimens suggest that the porphyrin component is distinct from the main 3–3*a* pigment.

Preparation of pigment fractions. In the isolation of larger quantities of material from *P. vulgaris* a somewhat different

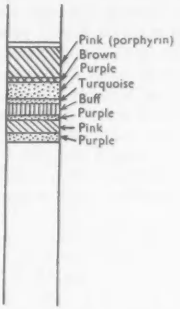

Fig. 3. Chromatogram on talc of acid extract of *Pinctada vulgaris* shells (partially purified; see text.

technique was used. Coarsely ground shells (500 g.) were extracted successively with 500 ml. 50% (v/v) H_3PO_4, acetone and 3N-HCl. The acetone was removed, and all extracts pooled, then diluted and allowed to percolate under suction through 1 kg. of pure talc in a large, squat column (20 × 10 cm.). The pigments were eluted with acetone. Zone 1 was rejected in the filtrate from the percolation. The

acetone eluate was now shaken with sufficient xylene to cause phase separation, the main pigments passing into the aqueous layer. A small amount of porphyrin passing into the xylene-acetone layer was recovered separately. The water phase was now concentrated under reduced pressure and placed on a column of talc, 12 × 3 cm. The resulting chromatogram was highly complex:

(Top)	Width of band (mm.)
Debris: ochre	
Blank	2
(Pink red fluorescence)	12
Brown	1
Purple	8
Turquoise	1
Buff	4
Violet	0·5
Pink	5
Violet	2

The column was developed with 15% (v/v) acetone in N-HCl. A brown and a pink band passed down (fraction *A*). The column was then extruded and cut. The violet and blue middle section, the brown layer above it and the top pink band of porphyrin were taken up separately in acetone and filtered (fractions *B*, *C* and *D*). They were left to evaporate at room temperature.

Fraction *A* on rechromatography gave an ill-defined separation into a lower brown and an upper pink band, probably identical with zone 2 of the initial chromatogram. Fraction *B* gave four zones; from above, violet, turquoise, unhomogeneous violet-brown, and a yellow trace, possibly extraneous, which washed out with 0·5 N-HCl. On cutting the column and redissolving in acetone, these fractions all showed bands at 587–556, 511–493 mμ., which were strongest in the violet top fraction. The lowest mixed band had an additional maximum at 465 mμ.

Fraction *D* was concentrated and dried *in vacuo*. The dry residue was extracted with ethyl acetate, and about a third of the porphyrin, estimated by fluorescence, passed into solution (Soret band: N-HCl, 405·5 mμ.; 3 N-HCl, 407·0 mμ.), and was extracted by shaking with N-HCl. The remaining ethyl acetate layer was now brown, with bands at 500, 480, and a trace near to 550 mμ. It was non-fluorescent. The residues of the original extract were treated with 3 N-HCl, and the remaining porphyrin passed into solution. A black insoluble residue remained on the filter. The two fractions of porphyrin from this rough separation were rechromatographed on paper by the technique of Nicholas & Rimington (1949), and are reported upon separately (Nicholas & Comfort, 1949).

The turquoise, red-fluorescing fraction (3*a* of initial chromatogram) was rapidly converted on standing to a yellowish, ether-soluble material, which could not be wholly re-extracted from acetone-ether mixture by shaking with 3 N-HCl. This yellow material was non-fluorescent and showed only a faint band close to 500 mμ.

The spectral characters of *Pinctada* fractions are summarized in Table 1.

All total extracts of *P. vulgaris* contained a brown, xylene- and amyl alcohol-soluble material which could be removed by partition from the acid solution.

Malleus regula Lk.

About one-quarter of one valve, including the area of hinge where traces of red fluorescence could be seen, was extracted with 100 ml. 2 N-HCl. Extracts of all parts of this shell were fluorescent, although fluorescence is not general in the intact shell.

Chromatography. The conspicuous blue-purple complex of *Pinctada* (bands 3–3*a*) was absent, but the chromatogram was otherwise similar. Band 1 contained more material than in *Pinctada*, but was identical in appearance and behaviour. The pink band (2) accounted for most of the visible colour, the porphyrin remaining as a sand-coloured zone at the surface of the talc, within the next (pink) band.

On development with 40% acetone to remove band 2, a brown fraction passed down and through it, appearing in the eluate just before the lower border of band 2. This fraction was distinct from the main body of band 2, and different in colour. The porphyrin band was small and rather faint.

Pinctada margaritifera

Acid extracts in HCl are reddish brown, with little or no porphyrin fluorescence. The olive shell becomes purple on contact with the acid. Large amounts of pigment can be recovered from the acid-treated shell by rewashing with acetone. The acid fraction showed bands at 501, 465·5 mμ. and the acetone fraction at 503 mμ., but when the acetone was removed by shaking with xylene and light petroleum, and the hypophase treated with excess 2 N-HCl, the spectra were identical. Another shell gave a different acid spectrum, the pigment being olive instead of reddish, and having bands at 481–513 and 555 mμ. Addition of a few drops conc. HNO_3 produced a shift to 464 and 504 mμ. The fractions were almost homogeneous on chromatography, except for a very small upper porphyrin zone.

Placuna sella

A deeply coloured shell was selected. Chromatography of the phosphoric extract was more difficult than in *Pinctada*, owing to the presence of brown bands overlapping the general pattern. The bands from above were porphyrin (olive), green, violet, pink, olive, violet, brick, broad pink. The

Table 1. *Characteristics of fractions obtained by chromatography on talc of crude extracts in phosphoric acid of shells of* Pinctada vulgaris

Fraction	Crude extract	1	2	3	4 and 5
Eluent	—	H_3PO_4 (33% v/v)	Acetone* (3% v/v)	Acetone* (10% v/v)	Acetone* (30% v/v)
Colour of eluate	Wine	Purple	Pink	Indigo violet	Pink
Abs. maxima (mμ.)	595, 553, 464	(500?) Very weak	545, 496	602, 500	614, 574, 551, 492, 464
Remarks	—	Single zone	Three zones eluted together	Two zones eluted together	Porphyrin: homogeneous

* Dilutions of acetone made in 33% (v/v) aqueous H_3PO_4.

lowest fraction on elution with 30% acetone was not homogeneous. It showed a strong double band at 493·5 and 466 mμ. The next fraction (eluated by 50% (v/v) acetone in 33% phosphoric acid) had a single band (480–505 max. 491·5 mμ.). With higher concentrations of acetone a further purplish pink band appeared high on the column (490–506, 553–567 mμ.) and moved down, followed by the porphyrin band. No clear-cut separation could be achieved on rechromatography. The lowest pigment was in all respects similar to the acid fraction of *P. margaritifera*, an entirely unrelated form.

Haliotis midae

The pigment is most easily extracted from immature shells. It is present in the outer layer of the shell, and also in the substance of the protoconch.

Shells extracted with conc. HCl give a port-wine coloured solution, changing to brown on dilution. The red solution shows bands at 560–530 and 472–455 mμ. On standing, it turns purple (max. 544, 495 mμ.). The Ehrlich (diazo) and Gmelin reactions are negative, and the pigments cannot be transferred to ether from neutral solution.

Chromatography of the acid extract on talc gives two main zones, and a yellow filtrate. These are red (below) and blue-green (above): the red zone becomes straw-coloured in acetone, but the red is restored by the addition of HCl. Only the red fraction shows an extremely faint red fluorescence; the blue pigment is non-fluorescent. It does not give a colour reaction with $NaNO_2$. No further colour change occurs with $FeCl_3$. On neutralizing total extracts and shaking with $CHCl_3$, however, traces of Lemberg's (1931) blue pigment can be extracted. The identity of the *Haliotis* pigments is discussed in a separate paper (Comfort, 1949*b*).

Turbo marmoratus

The main pigment of this species has been crystallized and fully investigated by Tixier (1947) under the name of turboglaucobilin. It is a blue, non-fluorescent bilitriene having a visible and an ultraviolet spectrum similar to those of glaucobilin. It was isolated for comparison with the talc chromatograms already obtained. Two specimens of *T. marmoratus*, which is probably identical with the *T. regenfussi* studied by Tixier, and which is named by him as a source of an identical pigment, were broken and extracted separately, half with acid ethanol (20% conc. HCl in ethanol) the pigments being transferred to $CHCl_3$ by the method employed by Tixier (1947), and half with 5 N-HCl for talc chromatography.

The direct chromatogram of the HCl extract gave three zones (from above, blue, violet, brown) corresponding to those found by Tixier (1947) on alumina from $CHCl_3$. Porphyrin was not detected. Solutions of rechromatographed turboglaucobilin ethyl ester in $CHCl_3$ were non-fluorescent in ultraviolet light.

Mixed chromatography

An attempt was made to relate the pigments of the *Pinctada* series to the known substances *Haliotis* blue and turboglaucobilin by chromatography of mixtures. In the mixed chromatogram of extracts of *Turbo*, *Haliotis cracherodii* and *Pinctada*, no evidence of identity was obtained, the pigments of the *P. vulgaris* series and the lower violet and brown bands of *Turbo* falling below the porphyrin zone, and *Haliotis* blue, turboglaucobilin, and the $CHCl_3$-soluble pigment of *P. margaritifera* above it (Fig. 4). Turboglaucobilin is more easily eluted by acetone than the porphyrin zone, and passes through it. Mixed chromatograms of *Pinctada*

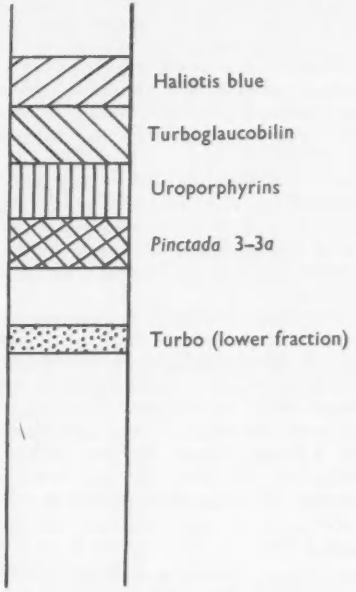

Fig. 4. Chromatogram (talc, N-HCl) of artificial mixture of shell pigments.

pigments with acid solutions of the products of the Gmelin reaction performed with conc. HNO_3 upon turboglaucobilin and upon biliverdin from cat bile showed no coincidence of zones. A solution in $CHCl_3$ of the products of this reaction upon turboglaucobilin was, however, spectrally similar to the $CHCl_3$-soluble fraction derived from *P. margaritifera*.

DISCUSSION

The pearl-mussel pigments, especially those of *Pinctada* and *Malleus*, can be separated into a number of fractions which show a superficially similar chemical behaviour. The number of substances present, the differences between related species and among individual shells of the same species and the insolubility of the pigments in organic solvents makes the purification and crystallization of individual substances extremely difficult, and pure specimens were not isolated. Except in the case of *Haliotis* blue, which will be discussed in a separate paper (Comfort, 1949*b*), the identity of these pigments has not been established. The *Pinctada* family of pigments exhibits no definite colour reaction comparable to that given by *Haliotis*

blue with nitrites (Schultz, 1931). This group does, however, behave as a consistent chromatographic series.

A rather similar family of pigments occurs in several archaeogastropod shells, especially among the red Trochidae and Fissurellidae, though chromatograms of these forms could not be repeated to our satisfaction owing to shortage of material. In other forms related to the porphyrin-producing groups, such as *Turbo* and *Haliotis*, we have been able to confirm Krukenberg's (1883) finding of bilitrienes but bilitrienes and porphyrins do not appear to coexist.

Mixed chromatography of *Pinctada* extracts with turboglaucobilin, with *Haliotis* blue and with the products of an incomplete Gmelin reaction performed on biliverdin from bile, shows no evidence of correspondence between known and unknown pigments.

The acid-soluble pigments of *Pinctada* have the following characteristics: they are indicators, changing to grey or greenish blue in alkaline solution; they are adsorbed from acid solution on talc in a consistent order, red falling lowest and violet highest upon the column; they are insoluble in organic solvents except acetone, ethanol and methanol, but they pass into amyl alcohol from acid solution on prolonged standing in a manner which resembles the behaviour of porphyrins, and strongly suggests esterification; they are non-fluorescent, and their visible absorption spectra are characteristic.

A close phylogenetic relationship exists between these pigments and shell porphyrins. They are found in typical purplish or reddish colour schemes, which seem to occur most commonly in primitive or unspecialized forms.

SUMMARY

1. The distribution of acid-soluble pigments in shells has been found to resemble that of the porphyrins.

2. The pigments can be separated by chromatography from aqueous acid upon talc. A characteristic series of pigments, which have not been identified, occurs in several bivalves and probably also in the Trochidae and Fissurellidae. Linear tetrapyrroles occur in the Turbinidae, where porphyrins are absent.

3. The pigments of higher molluscan shells are not extracted by acid, and appear to be chromoproteins. The possession of acid-soluble shell pigments is characteristic of the archaeogastropods, the lower bivalves, and the tectibranch opisthobranchs.

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Acid-soluble Pigments of Molluscan Shells

3. THE INDIGOID CHARACTER OF THE BLUE PIGMENT OF *HALIOTIS CRACHERODII* LEACH

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(*Received* 18 *November* 1948)

The investigation described in this paper was designed to establish the identity of the blue shell pigment of the abalone (*Haliotis cracherodii*), and to examine its relationship with other acid-soluble shell pigments.

The pigment was described by Krukenberg (1883), and has received considerable attention (Schultz, 1904, 1931; Kodzuka, 1921; Schultz & Becker, 1931; Lemberg, 1931). Workers have been divided between the later view of Schultz (1931) that it is an indigoid body, and that of Schultz (1904), Lemberg, Kodzuka and Krukenberg that it is pyrrolic.

Lemberg (1931) described the extraction of the pigment with acid ethanol. The extract was treated with chloroform followed by 0·05N-HCl, which removed a blue material (*B*), leaving a green

chloroform solution (*A*). Similar methods of extraction were used by other workers. In no case was the extract chromatographed. Although failing to behave as a glaucobilin (Lemberg, 1931) the blue pigment gave the pine-splinter reaction for pyrrole (Kodzuka, 1921) and a substance resembling a dipyrrylmethene on treatment with phosphonium iodide (Lemberg, 1931). The literature is reviewed by Lederer (1940).

EXPERIMENTAL AND RESULTS

Identity of material. The species figuring in the literature as *Haliotis californiensis* Sow. is almost certainly *H. cracherodii* Leach, of which *H. californiensis* is an uncommon variety. The results of Kodzuka (1921) refer to *H. gigas*. In the present study shells of *H. cracherodii* from California were used throughout.

Chromatography of the pigments of Haliotis cracherodii

Extraction. Extracts were prepared from coarsely broken shell: (*a*) with acid ethanol, for the preparation of Lemberg's (1931) *A* and *B* fractions; (*b*) with 5 N-HCl, for direct chromatography on talc by the method developed for the isolation of porphyrins (Comfort, 1949*a*); (*c*) by preliminary acid extraction, the pigment being concentrated by shaking with amyl alcohol, into which it passes from aqueous acid.

(*a*) When shells are extracted with acid ethanol, and the extract partitioned according to the instructions of Lemberg (1931) his *A* and *B* fractions are readily prepared. The *A* fraction, when further treated with conc. HCl, is removed almost completely from the $CHCl_3$ phase as a deep purple material showing bands at 661–586, 539 and 461 mμ. When chromatographed on talc, this extract gives a green-blue upper and a violet lower zone.

(*b*) Since our object was to compare the pigments of this species with those accompanying porphyrins in other forms, especially *Pinctada*, most of our extracts were made in 5 N-HCl and chromatographed upon talc from aqueous acid, using acetone-HCl or 0·5 N-NaOH as developer.

A medium-sized specimen (length 13 cm.) was coarsely broken, and as much as possible of the nacreous shell removed. It was macerated for 24 hr. with 500 ml. 5 N-HCl, and the blue extract decanted from green insoluble residue. A column of talc was packed from suspension in N-HCl by suction over cotton wool in a tube 20 × 2 cm. The extract was filtered and drawn through the column.

The bands of the undeveloped chromatogram are shown in Fig. 1*a*. The lower four fractions, eluted together by 10% (v/v) acetone in N-HCl, were shaken with amyl alcohol, which removed orange and yellow pigments having a positive Gmelin reaction, and left a green, Gmelin-negative hypophase. The upper blue and purple bands, eluted slowly by acid ethanol (20% HCl (v/v) in ethanol), gave the intense red colour with $NaNO_2$ which characterizes *Haliotis* blue. No ultraviolet fluorescence was detected in this chromatogram.

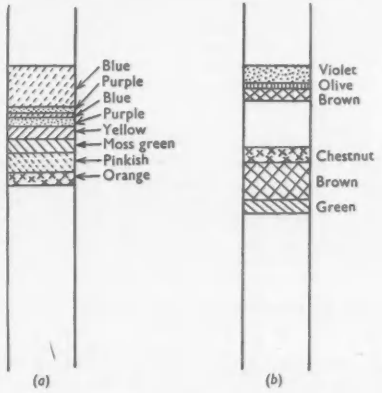

Fig. 1. (*a*) Undeveloped chromatogram on talc of acid extract of shell of *H. cracherodii*. (*b*) Partly developed chromatogram on alumina of ether-soluble fraction of *H. cracherodii* extract.

(*c*) A similar acid extract was prepared (see flow sheet, Fig. 2) by macerating two large shells with 300 ml. 7·5 N-HCl. The deep blue solution was filtered and shaken with 600 ml. amyl alcohol in small successive portions. The amyl alcohol solution, containing most of the pigment, was evaporated to dryness under reduced pressure, and the residue taken up in 20 ml. ethanol. Ether (20 ml.) was added, the mixture was placed overnight in the refrigerator, and then treated with excess 2 N-HCl. The blue pigment remained in the hypophase, leaving a greenish-yellow ether layer.

The ether solution was chromatographed on a column (prepared from a suspension in light petroleum) of alumina (Spence '0') in a tube 10 × 1 cm. The column was washed

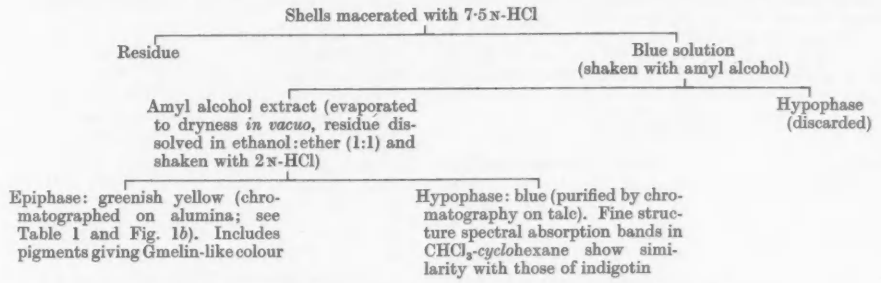
Shells macerated with 7·5 N-HCl
Residue
Blue solution (shaken with amyl alcohol)
Amyl alcohol extract (evaporated to dryness *in vacuo*, residue dissolved in ethanol:ether (1:1) and shaken with 2 N-HCl)
Hypophase (discarded)
Epiphase: greenish yellow (chromatographed on alumina; see Table 1 and Fig. 1*b*). Includes pigments giving Gmelin-like colour
Hypophase: blue (purified by chromatography on talc). Fine structure spectral absorption bands in $CHCl_3$-*cyclo*hexane show similarity with those of indigotin

Fig. 2. Flow sheet to illustrate separation of pigments from *Haliotis cracherodii*.

with several changes of ether, and showed the zones listed in Table 1 from above downward. Development with acetone 40% (v/v) in ether produced the chromatogram shown in Fig. 1*b*. The violet upper band was found to consist of the alkaline phase of the main blue pigment. The fractions eluted by acetone gave a positive Gmelin reaction.

Table 1. *Chromatogram on alumina of ether-soluble pigments from crude extract of* Haliotis cracherodii

Colour of zone	Width (mm.)
Moss green	3
Brick red	3
Orange	10
Buff	7

The acid-alcohol solution (hypophase of the initial ether partition) was freed of amyl alcohol by partial evaporation, and proved to be almost homogeneous on talc chromatography. This fraction was eluted with acid ethanol, again transferred to amyl alcohol and evaporated to dryness under reduced pressure. The residue was examined spectroscopically.

Ultraviolet spectroscopy and indigoid nature of Haliotis *blue*

The rechromatographed material described above was dissolved in $CHCl_3$-*cyclo*hexane, and its fine structure bands compared with those of the known indigos by means of a Hilger quartz spectrograph. The results are given in Table 2.

Table 2. *Ultraviolet absorptions of* Haliotis *blue and of indigotin in chloroform*-cyclo*hexane solution*

Absorption maxima (A.)	
Haliotis pigment	Indigotin
3570	3560
3370	3370
3226	3210
3070	3070
—	2856
—	2745

The similarity of these spectra affords strong evidence for the identity of the chromophoric groups in the two substances, and the conclusion appears justified that the *Haliotis* pigment contains an indigo closely related to, but not identical with, indigotin. The objections of Lemberg (1931), based on solubility considerations, do not contradict this conclusion although they suggest that the substance is not one of the known indigo pigments. Its solubility in acids is probably due to the presence of side chains attached to the indigo nucleus.

DISCUSSION

We have shown (Comfort, 1948, 1949*a*, *b*) that porphyrins are widely distributed among the Archaeogastropoda, but almost wholly absent from the species of *Haliotis* which we have examined. If *Haliotis* blue is a pyrrole derivative, as suggested by Lemberg (1931) and Kodzuka (1921), it might be held to represent an end product of the pigment cycle which gives rise to porphyrins in the Trochidae (Tixier, 1945), and to bilitrienes in the Turbinidae (Krukenberg, 1883; Tixier, 1947). If, on the other hand, Schultz (1931) was correct in regarding it as an indigoid body, it is the only substance of this kind known to occur in molluscs other than the Stenoglossa, many of which produce 6:6′-dibromoindigotin as a visceral and egg pigment.

Lemberg's objections to the findings of Schultz are threefold: that the pigment does not show the typical solubilities of indigo blue itself; that it is more basic than any known indigoid, including the dialkyl indigos; and that resemblances in the visible spectrum are not conclusive evidence of relationship between this body and the spectrally similar indigos cited by Schultz. (*C*-methylindigo 620, dibromoindigotin 623 mμ., Krüss & Oeconomides, 1883.)

The red material produced from *Haliotis* blue by nitrites in acid media bears some resemblance to that produced from bile pigments in the Gmelin reaction. Schultz (1931) regards the red colour as being due to isatin. When nitrite is added to a solution of *Haliotis* blue purified by chromatography, the development of the red colour requires a considerable excess of hydrochloric acid. With nitric acid the colour change is rapid, but not typical of the Gmelin reaction. The pigment becomes first violet and then red, with disappearance of the band at 622 mμ. and appearance of two new bands close to 500 and 550 mμ. Other fractions from the chromatogram show the characteristic sequence of colours given by bile pigments, but none produces a colour comparable with that of the nitrite reaction. The red material is very soluble in amyl alcohol, and only slightly soluble in chloroform. Its spectrum does not coincide with that of the Gmelin products, or of the purplish red solution produced by treating turboglaucobilin (Tixier, 1947) with nitric acid. Moreover, a similar colour appears spontaneously in acid solutions on standing, or very rapidly on the addition of hydrogen peroxide, in the presence of which the colour finally fades to pinkish orange.

The colour change of the *Haliotis* pigments in this case does not, therefore, coincide with that given by bilitrienes. It is far closer to the reaction described by Nencki & Sieber (1882) in the urorosein test. Herter (1908), Homer (1915), Watson (1938, 1939), Waldenström (1937) and Rimington, Holiday & Jope (1946) have discussed this test in some detail. Three main red pigments have been isolated from porphyrin-free urines: urorosein, indirubin (Watson, 1938, 1939; Rimington *et al.* 1946) and urocarmine (Fearon & Thompson, 1930). The solubilities of these pigments, and their spectral absorption bands, are compared in Table 3.

The end product of the action of sodium nitrite on *Haliotis* blue resembles urorosein both in these

Table 3. *Comparison of absorption spectra of urorosein and of the red derivative of* Haliotis *blue*

Substance	Solvent	Spectrum (abs. max. mμ.)	Solubility: Chloroform	Solubility: Amyl alcohol
Haliotis derivative	Amyl alcohol, conc. soln.	565–468	Slight	Marked
	Amyl alcohol, dilute soln.	522–492	—	—
	2 N-HCl, conc. soln.	559–550	—	—
	2 N-HCl, dilute soln.	540–490	—	—
Urorosein (Watson, 1939)	Amyl alcohol	555–557	—	Marked
	2·5 N-HCl	544–511	—	—
From indolylacetic acid (Homer, 1915)		552–531 505–492	—	—
From indolylaceturic acid (Homer, 1915)		579–539 502–485	— —	— —
Urocarmine (Fearon & Thompson, 1930)		No data	—	Marked

properties and in being reversibly decolorized by sodium acetate. Studies with the Beckman spectrophotometer gave density curves which varied somewhat from specimen to specimen, that given in Fig. 3 being typical. In acid solutions the curve closely resembles that of urorosein (Rimington *et al.* 1946), which is generally admitted to be an indole derivative.

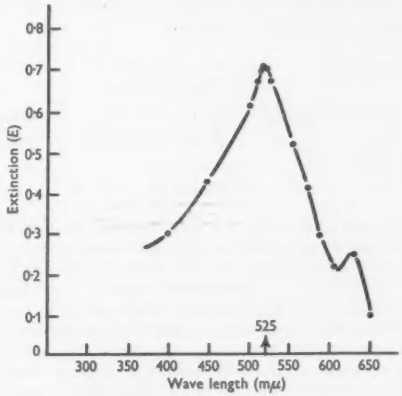

Fig. 3. Spectral absorption of red derivative (produced by addition of HNO_2) of *Haliotis* blue in acetone.

In the present paper, a comparison is recorded of the fine structure absorption in the ultraviolet of purified *Haliotis* blue and indigotin. The similarity observed is so marked as to justify the conclusion that *Haliotis* blue possesses an indigoid structure. The colour reactions observed with nitric and nitrous acids are reconcilable with this conclusion. The solubility of the *Haliotis* pigment in dilute hydrochloric acid indicates, however, that it must possess substituent groups modifying the character of the nucleus.

SUMMARY

1. The blue pigment of the shells of *Haliotis cracherodii* has been purified chromatographically. It has an ultraviolet absorption spectrum almost identical with that of indigotin, but differs from that substance in its solubility characteristics.

2. The reaction of the blue pigment with oxidizing agents results in a product which resembles urorosein in its spectral and chemical properties.

3. *Haliotis* blue is considered to possess an indigoid structure modified by the presence of substituent groups.

4. Other fractions derived from the same species contain pigments giving a reaction closely similar to the Gmelin reaction for bilitrienes.

NOTE

Added 16 *May* 1949

Since the publication of the preliminary results of this study, a fuller investigation of *Haliotis* blue has been made by Tixier & Lederer (1949), who have kindly made their findings available to us in manuscript. Their figures for the molecular weight and elementary composition of *Haliotis* blue methyl ester, purified by chromatography, are incompatible with an indigoid structure, and suggest a copromesobiliviolin. They regard the pigment as a bilidiene differing from the known pigments of this type in its spectral characters and in the fact that its zinc complex is non-fluorescent. A joint investigation of the interpretation of our findings and those of Tixier & Lederer will be undertaken, and its results published in a further communication.

Thanks are due to Prof. C. Rimington for the use of the Beckmann spectrophotometer and for access to unpublished work by him, and to Dr E. M. Jope, London Hospital Spectrographic Unit, Medical Research Council, for the spectroscopic study of *Haliotis* blue. The cost of the research was borne by the Yarrow Research Fund, London Hospital Medical College.

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Acid-soluble Pigments of Molluscan Shells

4. IDENTIFICATION OF SHELL PORPHYRINS WITH PARTICULAR REFERENCE TO CONCHOPORPHYRIN

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(*Received* 13 *December* 1948)

Particular interest has been attached to the nature of the porphyrins present in shells since Fischer & Jordan (1930) reported the occurrence in the marine mollusc *Pteria radiata* of a porphyrin, containing five carboxyl groups, which they named conchoporphyrin, and which was the only pentacarboxylic porphyrin known in nature. Fischer & Holt (1934) later compared it with a synthetically prepared pentacarboxylic porphyrin. The findings of other workers (Fischer & Haarer, 1932; Waldenström, 1937; Tixier, 1945) indicate, however, that only uroporphyrins I and III and traces of coproporphyrin are responsible for the pink fluorescence of molluscan shells.

One of us (Comfort, 1949) has recorded an extensive survey of the pigments present in molluscan shells, and has noted the phylogenetic distribution of porphyrin.

Having at our disposal a method of separating porphyrins, by partition chromatography, into groups depending upon the number of carboxyl functions in the molecule (Nicholas & Rimington, 1949; Rimington, 1949), we have examined as many porphyrin-containing shells of different species as possible, including *Pteria radiata* Lk., in the hope of confirming and extending Fischer & Jordan's (1930) observations. We have also had access to some of the original porphyrin material prepared by these authors (m.p. 270–273°) kindly provided by Prof. J. Waldenström of Uppsala, but we have obtained no evidence for the existence of a pentacarboxylic porphyrin.

METHOD

Porphyrins and other acid-soluble shell pigments are quantitatively adsorbed on talc; satisfactory separation of the individual porphyrins was not, however, achieved by this method. The acetic acid-ether technique for the extraction of ether-soluble porphyrins is difficult to apply to shell extracts on account of the large quantities of calcium salts.

Talc adsorption chromatography was used in order to separate the porphyrin as far as possible from the other shell pigments present, and finally the paper-partition method (Nicholas & Rimington, 1949) for the separation of the individual groups of porphyrins.

Technique of talc chromatography

Preparations of columns. Talc powder (pharmaceutical) was shaken with excess of 2N-HCl, and the suspension poured through a funnel containing some glass beads (to remove bubbles) into glass tubes 20 × 2 cm., the lower end of each being closed by a cotton-wool plug resting on a ceramic insulator. The suspension was then packed with the aid of suction.

Extraction of shells. The shells were broken and decalcified for 24 hr. in conc. HCl, or in a mixture of phosphoric acid (sp.gr. 1·75) with an equal volume of water. The extracts were filtered through glass wool to remove debris, adjusted to contain between 1 and 2·5N-acid by dilution with water, and poured into the tubes.

Chromatography. The extracts were drawn through the columns under suction. The chromatograms were either developed with increasing strengths of acetone in aqueous 3N-HCl, or dried for several hours over suction and extruded by air pressure from below, the fluorescent zones being cut out with a cover slip, or with a specially made glass knife.

Precautions. The adsorptive power of various samples of talc showed considerable variation, and the acidity of the solvent should not exceed 2·5N; stronger acids lead to blurring of bands and reversal of their order. Packing from acid suspension was found necessary, even with 'acid-washed' talc, to avoid cracking of the column when suction was applied.

The detailed appearance of the chromatograms and the spectral character of the non-porphyrin bands has been described in a previous paper of this series (Comfort, 1949). The porphyrin present was localized to one narrow pink fluorescent band, except in the case of *Pteria vulgaris*, where division of the porphyrin was obtained during the separation of a blue pigment; spectroscopy of the porphyrin fractions in acid solution showed in each, however, a Soret band consistent with uroporphyrin ($\lambda = 405$ mμ. in 2N-HCl).

Preparation of porphyrin for partition chromatography

The section of the talc columns containing the porphyrin, as shown by ultraviolet light, was cut out and placed in acetone containing 1% by volume of 3N-HCl. This eluted all the porphyrin together with some of the other pigments adjacent to it on the original talc column. The eluate was filtered through a sintered-glass funnel to remove the talc and the latter washed with HCl-acetone solution. After combining the washings with the first eluate, the acetone was removed under reduced pressure at room temperature. The solution, which was highly fluorescent in ultraviolet light, but varied in colour with individual shells from brown to violet, was then passed through a small talc column to remove the remaining non-porphyrin pigments and the inorganic salts which interfere with the partition chromatography.

This column, 3 × 0·5 cm., was packed from a suspension of talc in distilled water; under these conditions the porphyrin is adsorbed on the top of the column with the other pigments beneath it. The column was then washed with distilled water until the eluate was free from Cl^-. Any remaining non-fluorescent pigments were then removed by washing the column with increasing concentrations of aqueous NH_3, beginning with 0·01N, the porphyrin being finally eluted with 10N-NH_3 in which it descends as a narrow band and can be collected in approximately 0·1 ml. of solution which was applied to the paper chromatogram.

The specimen of Fischer's conchoporphyrin ester (m.p. 270–273°; Fischer & Jordan, 1930) was prepared for chromatography by hydrolysing it in approximately 7N-HCl for 24 hr. at room temperature. The HCl was removed in a vacuum desiccator over KOH and the porphyrin residue dissolved in 0·1 ml. of aqueous 10N-NH_3.

Partition chromatography

The partition chromatograms were run on strips of Whatman no. 1 filter paper (6 × 40 cm.) at a temperature of 21°, using a mixture of the 2:4- and 2:5-dimethylpyridines ('lutidine') as solvent, and in an atmosphere saturated with the solvent and water vapour.

Porphyrins are separated under these conditions into spots clearly visible by their pink fluorescence in ultraviolet light. It has been shown that the R_F values of the spots bear an inverse linear relationship to the number of carboxyl functions in the porphyrin molecule which they represent (Nicholas & Rimington, 1949).

The R_F values at 21° for representative porphyrins are recorded in Table 1.

Table 1. *R_F values of porphyrins in 'lutidine' at 21°*

Porphyrin	R_F values	No. of carboxyl functions
*Uroporphyrin	0·07 (0·12)	8
Coproporphyrin	0·5	4
Protoporphyrin (and other 2-carboxyl porphyrins)	0·75	2
Phylloerythrin	0·87	1
All esters	0·98	0

* Uroporphyrin characteristically gives two spots.

In order to avoid misinterpretation through slight changes in R_F due to variation in conditions, known markers were run on the strip in every case alongside the material under investigation.

RESULTS

The results which we have obtained with eight different types of shell and with Fischer's conchoporphyrin (Fischer & Jordan, 1930), stated to be derived from *Pteria radiata*, are recorded in Table 2.

Table 2. *R_F values and identification of porphyrins present in materials examined*

(The R_F value in brackets represents the additional uroporphyrin spot.)

Shell	R_F values of spots obtained	Porphyrin present
Fischer's 'conchoporphyrin' (*Pteria radiata*)	0·06 (0·11) 0·49	Uroporphyrin and coproporphyrin
Pteria radiata from S. Carolina	0·07 (0·12)	Uroporphyrin
Pinctada vulgaris (*Pteria vulgaris*)	0·07 (0·12) 0·49	Uroporphyrin and coproporphyrin
Trivia europœa	0·07 (0·12)	Uroporphyrin
Bulla sp.	0·08 (0·14)	Uroporphyrin
Placuna sella	0·05 (0·1)	Uroporphyrin
Umbonium australe	0·08 (0·13)	Uroporphyrin
Malleus vulgaris	0·06 (0·11)	Uroporphyrin
Gibbula cineraria	0·07 (0·11)	Uroporphyrin

DISCUSSION

The results confirm earlier evidence that the porphyrin present in shells is uroporphyrin, accompanied in some cases by smaller quantities of coproporphyrin. No evidence has been found for the existence of a penta- or hepta-carboxylic porphyrin.

The fact that the uroporphyrin does not behave as an entity on the chromatogram would point to the possibility of a seven carboxyl porphyrin (R_F 0·12) being present. This has been excluded on the grounds: first, that other samples of uroporphyrin of non-molluscan origin, considered pure by all other standards, behave identically; and secondly that the 'spot' of R_F 0·07, which represents the majority of the porphyrin, can be shown to decompose again into two spots (R_F 0·07 and 0·12) by rotating the chromatogram through 90° and re-running in the new direction with the same solvent.

There exists an element of doubt surrounding Fischer's identification of his shell material (Fischer & Jordan, 1930); his published papers deal specifically with two species of *Pteria*, viz. *Pteria radiata*, described as a Venezuelan species from which Fischer & Jordan (1930) obtained conchoporphyrin, and *Pteria vulgaris*, now called *Pinctada vulgaris*, which is widely distributed in the Indian Ocean and used in the button and fancy goods trade under the name of Persian lingah shell.

The specimens of *Pteria radiata* Lk.* which we have examined were from South Carolina and their identity was kindly established by Dr John Burch of Los Angeles who supplied them. We have also examined carefully identified *Pinctada* (*Pteria*) *vulgaris* from Bahrein on the Persian Gulf. These were obtained from the dealer who supplied Fischer with his specimens of *P. vulgaris*.

In view of the uncertainty, both chemical and zoological, surrounding Fischer's material, it would be difficult to express a definite opinion as to the existence of his 'conchoporphyrin', but the fact that we found his ester material, m.p. 270–273°, to be a mixture of uroporphyrin and coproporphyrin, casts doubts upon his claims. Certainly no pentacarboxylic porphyrin is present in any of the species of shell we have examined.

The uniformity which we find between the porphyrins of different groups of shells might have been predicted if it is assumed that they are end products of a closely similar or identical metabolic cycle. It is of interest that no porphyrins of the chlorophyll series have been encountered.

Although we have not been able to confirm the existence of conchoporphyrin, it may be mentioned that the technique of partition chromatography has revealed in other materials the existence of porphyrins presumably containing five and seven carboxyl groups respectively (Rimington, personal communication).

* A photograph of the shell may be obtained from the authors.

SUMMARY

1. Extracts of porphyrin-containing molluscan shells have been examined by adsorption and paper-partition chromatography.

2. The presence of uroporphyrin and, in one species, of coproporphyrin has been shown.

3. No evidence has been found for the existence in these shells of the pentacarboxylic porphyrin described by Fischer & Jordan (1930) under the name of 'conchoporphyrin'.

We wish to express our thanks to Prof. C. Rimington for his help and encouragement and for access to unpublished data, to Prof. J. Waldenström of Uppsala for a specimen of Fischer's conchoporphyrin, to Dr E. M. Jope, Medical Research Council Spectrographic Unit, London Hospital, for assistance with spectrographic measurements and to Miss A. Benson, for technical assistance. The work was financed by the Yarrow Research Fund (London Hospital) and by a grant from the Medical Research Council. The substance of this, and the foregoing three papers on molluscan pigments in this series, has been approved as part of a Thesis for the degree of Ph.D. in the University of London.

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Distribution and Nutritive Value of the Nitrogenous Substances in the Potato

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In a former communication, Chick & Cutting (1943) reported a series of experiments in which the nutritive value of the proteins and other nitrogenous substances of the potato was compared with that of casein and of the proteins in wheat. The criterion adopted was the ability to support growth in weanling rats fed on diets adequate in respect to other essential nutrients, but containing a sub-optimal amount of nitrogen derived solely from the food under test. Under these conditions, in which the nitrogen is the factor limiting growth, the relative rates of growth provide a basis for the comparison of the nutritive values of the mixtures of proteins (Chick, 1947; Slack, 1948*b*).

By the above method the nutritive value of the nitrogen of the potato was found to be somewhat superior to that of wheat nitrogen, although the digestibility was lower. This result was the more remarkable, since less than half of the nitrogen of the potatoes studied was present as protein, and about 60 % consisted of amides, nitrogenous bases and free amino-acids. The non-protein nitrogen contained in the expressed juice, after removal of the protein material by heat coagulation at pH 4, was unable to support growth. Nevertheless, the mixture of protein and non-protein nitrogen in the whole potato had a nutritive value at least equal to that found for the separated protein material.

The fact that the nitrogen of the potato, weight for weight, was as efficient as that of wheat for maintenance of nitrogenous equilibrium in adult man or animals, has been abundantly demonstrated (Rubner, 1879; Hindhede, 1913; Abderhalden, Ewald, Fodor & Rose, 1915; Rose & Cooper, 1917; Rubner & Thomas, 1918; Kon, 1928; Kon & Klein 1928). The reason why this should be so, seeing that 50 % or more of the potato nitrogen is non-protein in nature, puzzled Rubner 70 years ago. The puzzle remains unsolved, in spite of the advances in knowledge of proteins and nutrition which have been achieved since the period when Rubner was confronted with it (see p. 219).

Proteins of the potato

A protein having the properties of a globulin, to which they gave the name tuberin, was found by Osborne & Campbell (1895, 1896) in the expressed juice of the potato and was extracted from the residual pulp with NaCl solution. Since the protein materials from both sources had similar elementary compositions and precipitation limits with $(NH_4)_2SO_4$, it was concluded that they were the same. They could be purified by dialysis and heat coagulation took place irregularly between 56 and 76°. The purified material contained 16·24 % N. Neuberger & Sanger (1942), in an investigation of ten varieties of potatoes, the total N content of which varied from 1·16 to 1·95 % (on dry weight), found the proportion of protein N to vary from 28 to 51 % of the total soluble N. Of the non-protein N, 34 % (range 19·4–42·1 %) consisted of amide N, glutamine and asparagine, 10 % of α-NH_2-N (range 6·4–12·8 %) and 23 % of basic N (range 20–28 %). A residue of the total N in the potato amounting to about 10 % could not be extracted by methods which included extractions with water, salt solution, weak alkali, ethanol, ethanolic alkali and urea. This N was assumed to be protein in nature and was present mainly in the skin and cortex of the tuber. Groot, Janssen, Kentie, Oosterhuis & Trap (1947) found Osborne & Campbell's (1895, 1896) tuberin to be a mixture of two proteins which were precipitated from dilute press juice at different pH values. For the globulin precipitated at the higher pH they retained the name tuberin; for the other they suggested the name 'tuberinin'. Separation could also be made by electrophoresis of the press juice and by fractional precipitation with $(NH_4)_2SO_4$. The relative amounts of tuberin and tuberinin present were about 71/29. Jirgensons (1946) concluded that, owing to the labile nature of the proteins in potato juice, several different proteins could be separated according to the methods used.

The work reported in the present paper forms a continuation of that already published (Chick & Cutting, 1943), by a further study of the complementary nutritive action of the non-protein nitrogen of the potato sap for the protein tuberin. In the search for an explanation of this complementary effect, an analysis of these fractions for their amino-acid content was essential. Another aim was to recombine the different isolated nitrogenous fractions, including any insoluble protein that might be present, in an attempt to reconstitute the nitrogenous mixture as it exists in the intact tuber, an attempt which has proved only partially successful.

The difficulty experienced in maintaining fresh potatoes in a stable condition for a continued study led to a research on the changes in distribution and in nutritive value of the nitrogen, which takes place

under different conditions of storage and to the use, where possible, of dried material for the tests. With this work was combined an investigation of the relative nutritive value of the nitrogenous materials situated in different portions of the tuber.

EXPERIMENTAL

Materials and methods

Animal tests. The general technique employed was similar to that described previously, the criterion of nutritive value being the growth-promoting efficiency of the nitrogenous substances for weanling rats (Chick, 1942; Chick & Cutting, 1943).

Potatoes. The potatoes were of the 'King Edward' variety grown in the Fenlands. In many experiments in which nitrogenous fractions were investigated, the mixture of nitrogenous substances contained in the intact potato was used as a standard of comparison. When the potatoes were steamed, skinned, mashed and dried at a temperature not exceeding 40° in a current of air, the nutritive value of the N of the dried material was identical with that of freshly steamed, skinned potatoes (Chick & Cutting, 1943), and if the dried material was stored in closed tins in a cool cellar, no serious subsequent deterioration was observed.

Diets made with dried whole potato were kept in the dry state, and, immediately before feeding, water was added in about the proportion present in the original fresh potato. Diets containing as source of N the separated nitrogenous fractions, or mixtures of these which were uncooked, were mixed with an appropriate amount of water and cooked in a steamer for about 1 hr. The pudding-like material obtained was found convenient to feed.

Potato protein. The total heat-coagulable globulin of potato sap, the tuberin of Osborne & Campbell (1896), was separated from the juice expressed from raw, washed, minced potatoes by a method based on that of Kon (1928). After adjusting the pH of the press juice to 4·5 it was heated to 80° on a water bath with continuous stirring for about 1 hr. The precipitate was allowed to settle, was centrifuged, washed with water, ethanol, acetone and ether and finally dried on a water bath. In the best preparations it was a light grey powder, but, if allowed to take up any moisture during the removal of the organic solvents, it rapidly became discoloured and coarser in texture. Material prepared in this way contained about 5–10% moisture and 14–15% N on the dry weight; the ash content averaged about 4%.

The protein material, as prepared by the above method and referred to as tuberin in this paper, contains the total heat-coagulable proteins in the potato sap and includes the two globulins separated by Groot *et al.* (1947) and others, as well as by ourselves (see below). It does not, however, include the 10% of insoluble protein presumably combined with the residual pulp, according to the observation of Neuberger & Sanger (1942).

The N of this tuberin had an average digestibility coefficient of about 63% (range 56–70%). Even when great care was taken in its preparation the highest digestibility recorded was still considerably lower than the average coefficient (76%) for the N of the whole potato.

Crude tuberin. In order to minimize any damage to the digestibility of the tuberin, use was sometimes made of crude preparations of protein obtained by washing the centrifuged protein sludge with water only, and either incorporating this material directly into the diet or drying it at low temperature in a current of air. Such preparations contained about 14% N on the dry weight and had a digestibility coefficient of 77%, i.e. about equal to that of the whole potato.

Protein-free juice. The filtrate of the juice after removal of the precipitated tuberin contained 0·3 g. N/100 ml. It was mixed with the aqueous washings of the tuberin, concentrated *in vacuo* at 40°, adjusted to pH 3 and stored in the refrigerator until required. When fed alone or as the major source of N in the diet, it caused severe diarrhoea, but this could usually be prevented by adding to the diet about 1·5% of shredded filter paper to provide roughage. The N contained in this juice was nutritionally adequate for maintenance, but proved barely capable of supporting growth even when fed at a level of 3% on the dry weight of the diet.

Potato pulp. After juice had been expressed from the raw, minced potatoes, the pulp was washed by suspension in water and pressed as dry as possible, at which stage it contained about 50% water. It was then dried in a current of warm air and powdered in a hammer mill. The weight ratio of the pulp (dry weight) to the expressed juice was about 20/80; the pulp contained about one-fifth of the total N of the potato.

Chemical studies

Distribution of the nitrogen in the potato. The potatoes used contained from about 1·7 to 2·0% N on the dry weight, the amount present as protein varying from about 40 to 53% of the total. These values varied, however, with the age of the potatoes and conditions of storage; one sample contained 47% protein N when fresh and 43% after 10 months in the chamber at 5°.

In the sample used about one-fifth of the non-protein N in the juice was present as free amino-acids, two-fifths as amides and two-fifths as nitrogenous bases (see also Neuberger & Sanger, 1942; Chick & Cutting, 1943). The press juice from the washed, minced, raw potatoes usually contained about 0·45 g. N/100 ml.; about one-third of this was protein and about two-thirds non-protein N.

We were able to detect at least two proteins in potato press juice. When the diluted press juice, from which the starch had been largely removed by filtration through thick chain cloth and subsequent sedimentation, was adjusted to pH 4 and allowed to stand, a protein which may be called 'α-globulin' precipitated out, the separation being facilitated by gentle warming in an incubator at 30°. A second protein, 'β-globulin' was precipitated when the supernatant solution was boiled. In a sample of freshly dug potatoes the proportion of α- to β-globulin was about 30/70; in a sample of old potatoes after storage for 1 year, these values were reversed and were about 65/35. One specimen of β-globulin which was analysed contained 15·6% N.

The amino-acid composition of tuberin. The total heat-coagulable protein in the press juice, as prepared by the method of Kon (1928), was analysed by one of the authors (E.B.S.) for its content of the undermentioned essential amino-acids. The methods used were those of Gale (1946) for lysine, Tristram (1946) for valine, leucines, tyrosine and phenylalanine; Macpherson (1946) for arginine and histidine; Lugg (1938) for cystine and methionine; Hess & Sullivan (1945) for tryptophan and Rees (1946) for threonine. The protein-free filtrate, from which the tuberin had been removed, was concentrated *in vacuo*, the amino-acid fraction

separated by the procedure of Vickery (1925) and analysed for essential amino-acids by the above-mentioned methods.

The results, which were published recently (Slack, 1948*a*), are summarized for reference in Table 1. It will be seen that the tuberin is richer in the ten essential amino-acids determined than is the amino-acid fraction of the N.P.N. If it is assumed that all the protein in the potato has the same composition as tuberin, the amino-acid composition of the 'crude' protein (N × 6) of the whole potato can be calculated. The values obtained (column 3, Table 1) show the degree to which the content of essential amino-acids in the 'crude' protein of the whole potato is lower than in tuberin. The significance of this result is dealt with below when the nutritive value of the different nitrogenous fractions is discussed.

Table 1. *Amino-acid content of potato sap*

	Amounts of essential amino-acids expressed as g./16 g. N in		
	(1) Tuberin fraction	(2) N.P.N. fraction	(3) Mixture of 53 parts (1) with 47 parts (2)*
Phenylalanine	6·6	4·1	5·4
Leucines	17·5	4·3	11·3
Valine	6·1	3·3	4·8
Tryptophan	1·6	—	0·8
Threonine	5·9	1·1	3·7
Arginine	6·0	2·6	4·4
Histidine	2·2	1·1	1·7
Lysine	7·7	1·9	5·0
Cystine	2·1	1·2	1·6
Methionine	2·3	0·8	1·6

* The proportions in which the protein and N.P.N. were present in the sample of potatoes used.

The figures given by Groot (1945) are not strictly comparable with those obtained in the present work. Groot's values refer to 100 g. tuberin of 13·9% N content whereas those in the present work are calculated per 16 g. N (see Table 1). Nevertheless, except for lysine content, for which Groot's figure is much lower, the divergencies are not too great considering the difficulties of this type of analysis. It must also be remembered that the values given in this paper for the amino-acid composition of the tuberin and the N.P.N. are applicable only to the particular sample of potato studied for, even though tuberin itself should be of constant composition, the variations which are known to occur in the relative proportions of protein and N.P.N. are sufficient to cause great variation in the amino-acid content of the whole potato.

Feeding experiments

(*a*) *Effect of storage upon the distribution and nutritive value of the nitrogenous constituents of the potato* (*Exp.* 1*a*, *b*, *c*)

Samples of three batches of King Edward potatoes (6, 8 and 10) were dried, after steaming and removal of the skin, within 1 month of lifting. Other samples of the same batches were similarly dried after storage for several months, those of batch 6 after storage for 10 months in a chamber maintained at 5°, those of batch 8 for 5 months in a clamp in the open followed by a further 5 months in a chamber at 5°, those of batch 10 for 5 months in a clamp. Analysis of the potatoes of batches 6 and 8 after storage for 10 months showed a slight increase in the N content on the dry weight, indicating loss of non-nitrogenous material during storage, presumably due to respiration. In the case of the potatoes of batch 10, which had been stored for 5 months only, this change was not observed (Table 2).

The above six samples of dried potatoes were incorporated as source of N in six diets, nos. 72 and 73, 74 and 75 and 122 and 123, respectively. Potato starch was included where necessary to equalize the N contents of the diets; further details of the composition of the diets are given in Table 2. The animal tests with the first four of the above diets were made simultaneously (in Exp. 1*a*, *b*), those with diets 122 and 123 some time later (in Exp. 1*c*). In each case the diets were fed to comparable groups of 6–8 litter-mate weanling rats for a period of 7 weeks; the food intake and the economy of utilization of the N eaten was measured during 1 week in

Table 2. *Composition of materials and diets used in Exp.* 1

(The proportions of constituents in the diets are given in parts by weight.)

Materials	Batch no.	State of potatoes	Moisture (%)	N (% of dry wt.)	Protein N (% of total N)	Exp. 1*a* Diet 72	Exp. 1*a* Diet 73	Exp. 1*b* Diet 74	Exp. 1*b* Diet 75	Exp. 1*c* Diet 122	Exp. 1*c* Diet 123
Dried King Edward potato	8	Newly lifted	12·6	1·88	42·8	100	—	—	—	—	—
	8	Stored 5 months in clamp + 5 months at 5°	10·9	2·04	37·3	—	91	—	—	—	—
	6	Newly lifted	12·2	1·82	47·2	—	—	100	—	—	—
	6	Stored 10 months at 5°	12·1	1·86	42·9	—	—	—	98	—	—
	10	Newly lifted	10·7	1·96	—	—	—	—	—	93	—
	10	Stored 5 months in clamp	10·7	1·96	—	—	—	—	—	—	93
Potato starch			10 (approx.)	0·01	—	—	7	—	2	—	—
Hardened arachis oil						4	4	4	4	4	4
Salt mixture*						2	2	2	2	2	2
Cod liver oil						1	1	1	1	1	1
Average N content of diet (% of dry wt.)†						1·73	1·70	1·66	1·65	1·78	1·80

* McCollum, Simmonds & Pitz (1917).

† Over whole 7-week period of the tests.

Exp. 1*a*, *b* and during 2 weeks in Exp. 1*c*. Average growth curves of the rats in all three experiments are presented

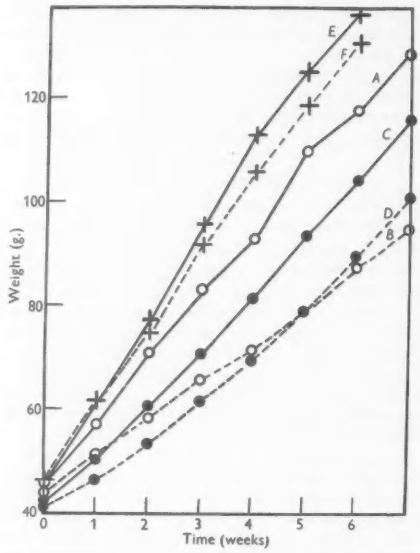

Fig. 1, Exp. 1*a–c*. Average weight curves of rats maintained on diets containing nitrogen from potatoes dried as follows:

Curve	Potato sample	Dried	Diet	N in diet (% of dry wt.)
A	KE 8	Freshly dug	72	1·73
B	KE 8	Stored 10 months	73	1·70
C	KE 6	Freshly dug	74	1·66
D	KE 6	Stored 10 months	75	1·65
E	KE 10	Freshly dug	122	1·78
F	KE 10	Stored 5 months	123	1·80

together in Fig. 1. In each case the rats, having diets containing potatoes dried immediately after lifting, had a higher rate of weight increase than those receiving potatoes dried after storage, although there was variation in the extent of the difference. The greatest deterioration was observed in the potatoes of batch 8 after storage for 5 months in a clamp, followed by a further 5 months in a chamber at 5°; there was a smaller but considerable decline in the nutritive value of batch 6 stored for 10 months at 5°. The average weekly weight increase over a 7-week period of the rats receiving fresh and stored samples of these two batches were, respectively, 18·4 and 13·5 g. (batch 8) and 16·5 and 14·4 g. (batch 6). With the potatoes of batch 10, which had been clamped for 5 months only, the deterioration was less marked. Nevertheless, the economy with which the N of this batch was utilized was considerably reduced by the storage, as indicated in Table 3, which gives the average performance/rat/week on each of the six diets. The same was true of the potatoes of batch 8, but was not observed with those of batch 6 during the actual week of measurement of the utilization.

These results indicate that the changes taking place in the nitrogenous constituents of the potato during storage, lead to a deterioration in their nutritive value.

(b) Nutritive value of the nitrogen of the whole potato with and without the peel, compared with that of various separated nitrogenous fractions fed alone or in various combinations

Exp. 2. Comparison of the nutritive value of the nitrogen of whole potato, potato minus skin and potato minus skin and outer cortex. The skin and outer layers of the potato are richer in total N than the inner layers, and also contain a higher proportion of insoluble N than the rest of the tuber (Neuberger & Sanger, 1942). In one sample of potatoes, examined soon after lifting, the skin accounted for 7·6% of the dry weight of the tuber and for about 10% of the total N. Thus it seemed desirable to determine whether the N contained in the skin and outer layers was of greater nutritive value than that in the inner parts, or had any supplementary value for this. Comparison was therefore made of the growth-supporting value of diets in which the N was supplied by (*a*) steamed whole potato, (*b*) the same potatoes steamed after removal of the skin, and (*c*) potatoes peeled before steaming to remove the skin and a layer about 2 mm. deep of the cortex. The potatoes used for the test (batch 6) were

Table 3. *Food intake and economy of utilization of nitrogen on diets nos.* 72–75 *and* 122, 123

(Average figures/rat/week.)

Exp. no.	Diet no.	Batch no.	State of potatoes	No. of rats	N in diet* (%)	Dry food eaten (g.)	N eaten (g.)	Average wt.* increase (g.)	Coefficient† of (apparent) digestibility of N	Wt. increase (g./g. N eaten)	Wt. increase (g./100 g. dry food eaten)
1*a*	72	8	Newly lifted	8	1·72	78·0	1·34	17·0	—	12·7	21·8
	73	8	Stored	8	1·67	58·7	0·98	7·7	—	7·9	13·2
1*b*	74	6	Newly lifted	8	1·64	71·7	1·17	9·6	—	8·2	13·4
	75	6	Stored	8	1·67	71·4	1·20	10·5	—	8·8	14·7
1*c*	122	10	Newly lifted	6	1·78	85·3	1·51	18·7	76·9	12·4	21·9
	123	10	Stored	6	1·80	81·6	1·47	14·0	78·9	9·5	17·2

* Average values during periods in which food intake was measured: Exp. 1*a*, fifth week; Exp. 1*b*, sixth week; Exp. 1*c*, third and fourth weeks.

† $\frac{\text{N eaten (g.)} - \text{N in faeces (g.)}}{\text{N eaten (g.)}} \times 100.$

steamed and dried within 2 months of lifting and had been stored in a clamp in the open.

Table 4. *Composition of materials and diets used in Exp.* 2

Material	Dry matter (%)	N (% of dry wt.)	Diet 50 (g.)	Diet 51 (g.)	Diet 52 (g.)
Steamed whole potato (batch 6)	22·2	1·72	500	—	—
Steamed potato minus skin	22·0	1·67	—	500	—
Steamed potato minus skin and outer cortex	21·2	1·63	—	—	500
Maize starch	90	0·03	8	5	—
Hardened arachis oil			4	4	4
Salt mixture*			2	2	2
Cod liver oil			1	1	1
Average N content of diet (% of dry wt.)†			1·54	1·56	1·58

* McCollum *et al.* (1917).
† Over whole period of test, 7·5 weeks.

Details of the three diets used (50, 51 and 52) are given in Table 4. They were fed to three groups of litter-mate rats for 7·5 weeks and measurements of food intake and of digestibility of the N were made during four successive weekly periods.

From Fig. 2 it is seen that the best growth rate was obtained on diet 52 which contained the inner portions only of the potato, the average weekly weight increase over the whole period of the test being 13·9 g. The corresponding figures for the diets containing the skinned and unskinned potatoes were 12·7 and 11·8 g. respectively.

In this, as in all cases involving the nitrogenous material in the potato, it is not possible to speak of the 'protein efficiency ratio', or the weight increase/g. protein ingested, seeing that so great a proportion of the N is of a non-protein nature. The ratio, weight increase (g.)/N ingested (g.) may, however, be substituted. The values of this ratio corresponding to diets 50, 51 and 52 for the 4-week period in which the food intake was measured were respectively 9·5, 10·2 and 11·4 (Table 5). The inclusion of the N of the potato skin and outer cortex in diet 50 resulted in a lowering of the digestibility of the N, the coefficient being 74·2% as compared with 77·8% for diet 51 and 78·6% for diet 52.

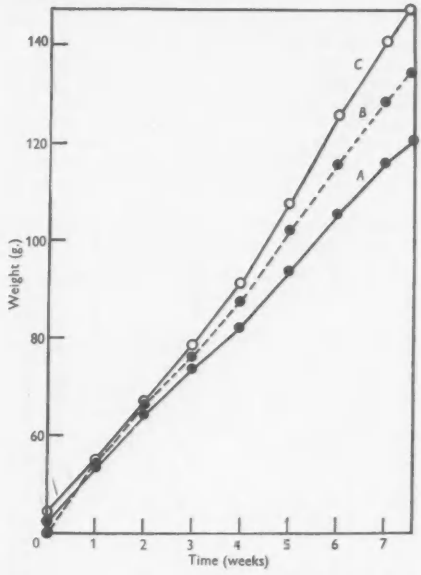

Fig. 2, Exp. 2. Average weight curves of eight rats maintained from weaning on diets containing 1·54–1·58% N (on dry weight) derived from potatoes (sample KE 6): *A*, potatoes dried after steaming with skin retained (diet 50); *B*, potatoes dried after steaming, skin removed (diet 51); *C*, peel to a depth of about 2 mm. removed from the potatoes before steaming (diet 52).

The slightly lower digestibility of the N in diet 51, which contained the potato skin, was not considered responsible for the inferior growth-supporting capacity of this diet. This conclusion was confirmed by the results of a further

Table 5. *Exp.* 2. *Food intake and economy of utilization of nitrogen on diets nos.* 50–52

(Average figures/rat/week.)

Diet no.	Source of N	No. of rats	N in diet* (%)	Dry food eaten (g.)	N eaten (g.)	Faeces passed (dry wt., g.)	N in faeces (g.)	N digested (g.)	Coefficient† of (apparent) digestibility of N	Average wt. increase (g.)	Wt. increase (g./g. N eaten)	Wt. increase (g./g. N digested)
50	Whole potato (batch 6)	8	1·53	71·1	1·09	5·42	0·281	0·809	74·2	10·4	9·5	12·9
51	Whole potato (batch 6) minus skin	8	1·55	74·5	1·15	4·82	0·255	0·896	77·8	11·8	10·2	13·2
52	Whole potato (batch 6) minus skin and outer cortex	8	1·58	80·3	1·27	5·40	0·271	0·997	78·6	14·4	11·4	14·3

* Average during 4-week period in which food intake was measure .

† $\frac{\text{N eaten (g.)} - \text{N in faeces (g.)}}{\text{N eaten (g.)}} \times 100.$

Table 6. *Composition of materials and diets used in Exps.* 4, 5 *and* 6

(The proportions of constituents in diets are given in parts by weight. Dried potato diets (35*A*, 73*A*, 107, 107*A*) before feeding were mixed water in amount about equal to that present in the fresh potato; other diets were mixed with water and steamed for 1–1·5 hr.)

Material	Exp. 4					Exp. 5					Exp. 6					
	Moisture (% approx.)	N (% of dry wt.)	Diet 35*A*	Diet 36*B*	Diet 39	Moisture (%)	N (% of dry wt.)	Diet 73*A*	Diet 76	Diet 77	Moisture (%)	N (% of dry wt.)	Diet 107	Diet 107*A*	Diet 108	Diet 109
Potato (dried)	11	1·93	93	—	—	12·0	1·97	91	—	—	+skin 10·7	1·96	104	—	—	—
											−skin 11·4	2·02	—	93	—	—
Potato protein (tuberin)		11·85 (air-dry)	—	—	13·5	6·2	14·0	—	13	10	crude 6·8	11·1	—	—	3·7	8·8
Whole press juice (concentrated)	80	1·32–1·97* (g./100 ml.)	—	100	—											
Protein-free juice (concentrated)			—	—	—						(100 ml.) 80·8	1·95	—	—	39	46·5
Potato pulp (dried)		0·91	—	—	—	15·6	0·48	—	—	101	crude 13·9	0·81	—	—	95·5	—
Potato starch							0·01	7	91	—			—	—	—	—
Maize starch	10	0·03	—	55–90†	80			—	—	—			—	—	—	—
Hardened arachis oil			4	3–4·5†	4			4	4	4			4	4	4	4
Salt mixture‡			2	1·5–2·3	2			2	2	2			2	2	2	2
Cod liver oil			1	1	1			1	1	1			1	1	1	1
Filter paper			—	1·2–1·4	—			—	—	—			—	—	—	—
Average N content of diet §			1·70	1·80	1·81			1·65	1·69	1·67			1·80	1·77	1·74	1·79

* According to degree of concentration.
† According to N content of concentrated juice.
‡ McCollum *et al.* (1917).
§ Over whole period of test: Exp. 4, 6 weeks; Exp. 5, 6 weeks; Exp. 6, 7 weeks.

Table 7. *Exps.* 4, 5 *and* 6. *Food intake and economy of utilization for growth of the nitrogenous substances present in the whole potato and their different fractions*

(Average values/rat/week.)

Exp. no.	Diet no.	Source of nitrogen	No. of rats	N in diet* (%)	Dry food eaten (g.)	N eaten (g.)	Faeces passed (dry wt. g.)	N in faeces (g.)	N digested (g.)	Coefficient of (apparent) digestibility of N†	Average wt. increase* (g.)	Wt. increase (g./g. N eaten)	Wt. increase (g./100 g. food eaten)
4	35*A*	Dried potato (batch 1, without skin)	5	1·70	64·3	1·10	5·60	0·269	0·83	75·4	12·4	11·3	19·3
	36*B*	Press juice	5	1·80	56·1	1·02	3·91	0·173	0·85	83·1	9·7	9·5	17·3
	39	Tuberin	4	1·81	54·5	0·99	4·07	0·355	0·63	64·0	10·9	11·0	20·0
5	73*A*	Dried potato (batch 8, without skin)	2	1·65	89·1	1·47	5·72	0·306	1·16	79·2	14·0	9·5	15·7
	76	Tuberin	3	1·67	69·3	1·55	5·33	0·503	0·65	56·4	9·6	8·3	13·8
	77	3/4 from tuberin 1/4 from washed pulp	3	1·66	59·3	0·98	6·58	0·515	0·47	47·5	6·7	6·8	11·2
6	107	Dried potato (batch 10, with skin)	5	1·82	57·6	1·05	5·19	0·249	0·80	76·3	7·6	7·2	13·2
	107*A*	Dried potato (batch 10, without skin)	5	1·78	62·6	1·11	4·62	0·259	0·85	76·8	11·2	10·1	17·9
	108	1/5 from crude tuberin 2/5 from unwashed pulp 2/5 from protein-free juice	5	1·74	55·3	0·96	5·23	0·297	0·67	69·2	5·8	6·02	10·5
	109	1/2 from crude tuberin 1/2 from protein-free juice	5	1·78	56·7	1·01	3·74	0·297	0·71	70·6	7·9	7·81	13·9

* Average during period in which food intake was measured: Exp. 4, 6 weeks; Exp. 5, 3 weeks; Exp. 6, 2 weeks.
† $\frac{\text{N eaten (g.)} - \text{N in faeces (g.)}}{\text{N eaten (g.)}} \times 100$.

experiment (Exp. 6, Table 7) made for a different purpose, in which the control tests with whole potatoes included trials of those dried with and without the skin (diets 107 and 107*A*, Table 6). The performance of the rats receiving the former was markedly inferior to that of those receiving the dried, skinned potatoes as regards weight increase and utilization of N and of total calories. The average weekly weight increases over a 7-week period were respectively, 8·2 and 10·8 g.; the ratio, weight increase (g.)/N ingested (g.), over a 2-week period in which food intake was measured, was respectively, 7·2 and 10·1. The digestibility coefficients of the nitrogen in the two diets were 76·3 and 76·8% respectively.

Exp. 3. *The nutritive value of the nitrogen in the protein-free juice fed alone, or mixed with tuberin, compared with that of the nitrogen of the whole potato.* A diet was prepared in which the nitrogen was provided by the protein-free juice of the potato; it consisted of 95 parts of concentrated juice containing 1·73% N and 20% dry matter, together with potato starch 71, hardened arachis oil 4, salt mixture 2, and 1 part each of cod liver oil and shredded filter paper; the average N content on the dry weight was 1·9%. It was fed to a group of six young rats. After 2 weeks on this regime, during which there was no appreciable gain in weight, the proportion of juice in the diet was increased to raise the N content to 2·3% on the dry weight without effect and in the following week to 3·0%, at which level the animals gained an average weight of 4·3 g. When, however, at the end of the fourth week, three of the animals were given a similarly composed diet containing 1·7–1·8% N on the dry weight, provided by a combination of tuberin with protein-free sap, to provide N from these in the proportion in which they were present in the whole press juice (ratio 1:2), the average weekly weight increase during the following 2 weeks was 9·1 g. (Fig. 3, Curve B_1). When, during the next 3 weeks, the proportion of protein to N.P.N. was adjusted to that in the sample of whole potato used (ratio 44/56), the average weekly weight increase was 6·8 g. (Fig. 3, Curve B_2). These results show the supplementary relation of the N.P.N. for that of tuberin, seeing that on a diet containing the same amount of N derived from the protein alone the average weekly weight increase was 9·6–9·7 g. (Table 7, Exps. 4 and 5). The remaining three rats in Exp. 3 were given a similarly composed diet containing 1·7% nitrogen provided from the whole potato. During the following 5 weeks steady growth occurred with an average weekly weight increase of 19 g., showing that in the case of the whole tuber the mixture of protein and N.P.N. nitrogen was even more efficient.

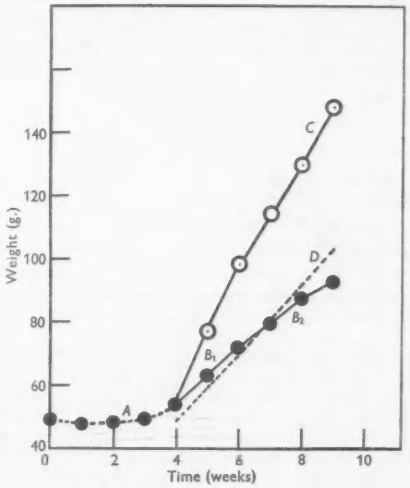

Fig. 3, Exp. 3. Average weight curves of weanling rats maintained on diets containing N derived from: *A*, protein-free press juice (1·9–3·0% N on dry wt.); B_1, tuberin N and non protein N in proportion of 1/2; B_2, in proportion of 44/56 (1·7–1·8% N on dry wt.); *C*, whole dried potato (minus skin, 1·7% N on dry wt.); *D*, tuberin (1·7% N on dry wt.) from Exp. 5.

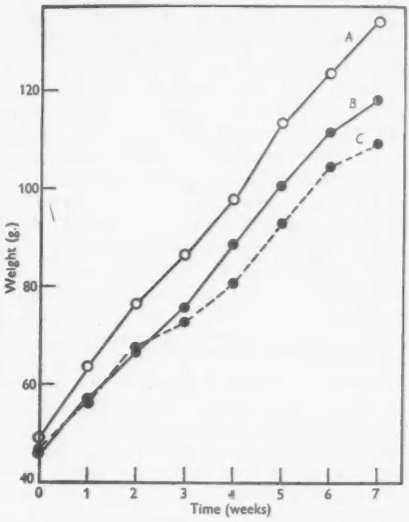

Fig. 4, Exp. 4. Average weight curves of four to five rats maintained from weaning on diets containing 1·7–1·8% N (on dry wt.) derived from *A*, whole dried potato (diet 35*A*); *B*, tuberin separated from the press juice (diet 39); *C*, whole press juice (diet 36*B*).

Exp. 4. *The nutritive value of the nitrogen (protein and non-protein) in the expressed whole juice, and of that of the protein (tuberin) separated from the juice, compared with that of the nitrogen of the whole potato.* Diets 36*B*, 39 and 35*A*, made to contain about 1·7–1·8% N (on the dry weight), derived from the sources given in the above heading, were fed to three comparable groups of 4–5 litter-mate rats from weaning (see Table 6). The results of this experiment are summarized in Table 7, and the average growth curves of the rats in the three groups are given in Fig. 4.

The performance of the rats receiving the whole dried potato as source of N was again better than that of those receiving the mixture of protein and N.P.N. as these are present in the whole press juice, the average weekly weight increases over a period of 6 weeks being 12·4 and 9·7 g.

respectively. The digestibility of the N in the latter, which contained simpler nitrogeneous material, was naturally higher (coefficient 83%) than that of the former (coefficient 75·5%). The N in the separated tuberin had also a lower digestibility than that of the whole potato, and the weekly weight increase (11·0 g.) was also lower. The ratios, weight increase (g.)/N consumed (g.), were similar for the whole potato (11·3) and tuberin (11·0) and somewhat lower (9·5) for the press juice.

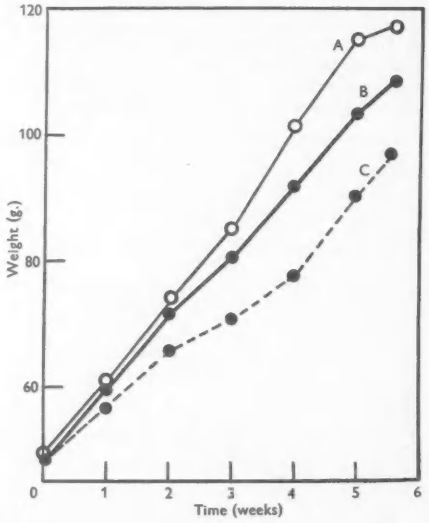

Fig. 5, Exp. 5. Average weight curves of two or three rats maintained on diets containing 1·65–1·70% N (on dry wt.) derived from potato, sample KE 8: *A*, whole potato dried without skin (diet 73*A*); *B*, separated protein (tuberin; diet 76); *C*, $\frac{3}{4}$ from tuberin, $\frac{1}{4}$ from washed pulp (diet 77).

The results with diets 36*B* and 39 demonstrate again the supplementary effect of the N in the protein-free fraction of the sap for that of the tuberin fraction.

Exps. 5 *and* 6. These were in essentials repetitions of Exp. 4, but more care was taken in the separations of the different fractions. In Exp. 5 all the fractions were more thoroughly washed; in Exp. 6 all processes were speeded up and washing of the fractions omitted. The N in the three diets used in Exp. 5, which contained 1·65–1·7% N on dry weight, was provided by whole potato dried after removal of the skin (diet 73*A*), or by washed dried tuberin (diet 76), or by a mixture of tuberin and the washed pulp left after pressing the juice from the raw minced potato (diet 77, in which the pulp provided about one-quarter of the N, a proportion similar to that provided by the pulp in the whole potato). All these materials were derived from the same batch of King Edward potatoes, but there was an interval of 3–6 months between the preparation of the whole dried potatoes and that of the tuberin and pulp, during which the potatoes were kept in a chamber at 5°.

The performance on the diet containing the whole potato was again the best; the average weekly weight increase over a period of 6 weeks was 10·1 g.; the ratio weight increase (g.)/N eaten (g.) during 3 weeks was 9·5; the coefficient of digestibility 79·2. The corresponding figures for the rats receiving their N from tuberin were 10·5 g., 8·3 and 56·4%, and for those receiving it from the combination of tuberin and pulp 8·6 g., 6·8 and 47·5%. The above values for the economy of utilization of the N are certainly influenced by the low digestibility of the N in the tuberin and the pulp.

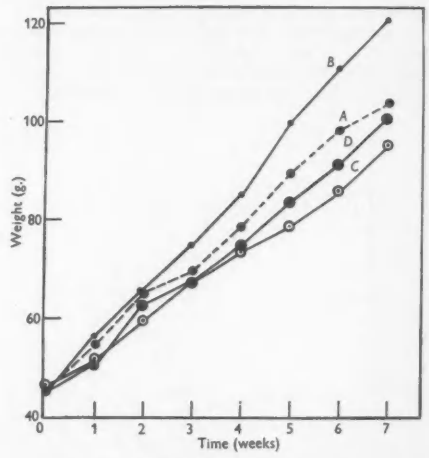

Fig. 6, Exp. 6. Average weight curves of five rats maintained from weaning on diets containing 1·74–1·82% N (on dry wt.) derived from potato sample KE 10: *A*, whole potato steamed and dried with skin (diet 107); *B*, whole potato dried without skin (diet 107*A*); *C*, $\frac{1}{5}$ from crude tuberin, $\frac{2}{5}$ from unwashed pulp, $\frac{2}{5}$ from protein-free press juice (in approximate proportions as present in the whole potato; diet 108); *D*, $\frac{1}{2}$ from tuberin, $\frac{1}{2}$ from protein-free press juice (diet 109).

In Exp. 6 the separation of the materials was effected as quickly as possible to minimize any changes that might follow this interference with the tissues. Neither the protein separated from the sap, nor the residual pulp, was washed; both were quickly dried at low temperature immediately after separation. In order to make the comparison with the whole potato more exact, tests were made with potatoes dried without removal of the skin (diet 107), seeing that in the preparation of the pulp the skin of the potato was included. The composition of the diets fed to the different groups of rats is set out in Table 6. In diet 108 the fractions of the N contributed by the tuberin ($\frac{1}{5}$), the protein-free juice ($\frac{2}{5}$) and the pulp ($\frac{2}{5}$), were arranged to reproduce approximately the fractions contributed by these constituents in the whole potato. The average weekly weight increase (6·9 g.) of the rats on diet 108 over the 7 weeks of the test was, however, significantly less than that of the animals on diet 107 receiving their N from the whole potato with its skin (8·2 g.). In diet 109 the N was contributed equally by the crude tuberin and the protein-free juice, an analysis of the

whole dried potato by Stutzer's (1880)method having shown that in this sample about 50% of the N was in the form of protein. On this diet the average weekly weight increase was 7·9 g., a value which should be compared with that of 10·8 g. achieved on the diet containing the whole potato after the removal of the skin (see Fig. 6).

The values for Exp. 6 in Table 7, which refer only to the period of 2 weeks during which measurements of the food and N intake and of the digestibility of the N were made, show the lowered degree of digestibility and of utilization of N when the pulp was included in the diet. The mixture of protein and N.P.N. in diet 109 also functioned less efficiently than the mixture in the whole potato after removal of the skin (diet 107*A*), with which in this case the comparison should strictly be made. Nevertheless, the results show clearly the existence of a nutritionally advantageous interaction between the protein and N.P.N. of this diet.

In all the above tests the N in the skinned whole potato was more economically used than any other fraction or combination of fractions except, possibly, the sample of tuberin tested in Exp. 4. Some possible explanations of this fact are discussed below.

DISCUSSION

The main experimental findings of the present work are summarized in Tables 1, 3, 5 and 7, and it is mainly around these results that the discussion is centred. One conclusion that may immediately be drawn concerns the extreme lability of the nitrogenous constituents of the potato, both as regards their chemistry and their nutritive value. The deterioration in nutritive value on storage of the potatoes tested in Exp. 1*a*–*c* (Table 3) is obviously in keeping with the changes in chemical composition reported on p. 212. From these findings alone it is obvious that any attempt to collate a series of results obtained on such labile material over a period of some years will meet with considerable difficulty. The task is made less easy when it is realized that the mildest chemical manipulations of the experimental material may produce changes profoundly affecting the nutritive value of its nitrogenous constituents. The potato tuber is a living, changing system and the processes involved in the separation of its constituents, no matter how carefully operated, seem to bring about unknown changes detrimental to nutritive value. Thus in Exp. 6 the nitrogen of a recombination of tuberin, protein-free juice and potato pulp had a nutritive value appreciably lower than that of the corresponding whole potatoes from which they had been prepared; the digestibility of the nitrogen of the reconstituted potato was only 69·2 as compared with 76·3% for the nitrogen of the whole potato.

The digestibility coefficients of the nitrogen of the preparations of tuberin used throughout the series of experiments were lower than that of the whole potato nitrogen, the mean value being 63% (range 56–70%) as compared with the mean value of 76% (range 75–79%) for whole potato. Nevertheless, it seems doubtful whether a difference in digestibility of this order can entirely account for the equal or superior nutritive value of the mixture of protein and non-protein nitrogen in the whole potato compared with that of tuberin.

The complementary action in nutritive effect between the protein and non-protein nitrogen in the potato is clearly shown in the present work. In Exp. 3, the non-protein nitrogen in the expressed sap was shown to possess no growth-supporting capacity when incorporated in a diet to the extent of 2% on dry weight. Nevertheless, when one-quarter of this nitrogen was replaced by tuberin nitrogen, in a diet containing 1·7–1·8% nitrogen, growth was promptly initiated at a rate not inferior to that supported by a similarly composed diet containing nitrogen derived solely from tuberin (see Fig. 3). A similar result can be derived from Exp. 4, where rats, which received their supply of nitrogen as the mixture of protein and non-protein nitrogen present in the expressed sap (diet 36*B*), gave a performance of the same order as that of litter mates whose diet (39) contained a similar proportion of nitrogen from tuberin (see Table 7 and Fig. 4).

No supplementary action could be demonstrated between tuberin and any insoluble protein remaining in the potato pulp, either when these two fractions were fed directly in combination as in Exp. 5 or compared indirectly as in Exp. 2. In the latter experiment the removal of the skin and outer cortex, which are the parts richest in the insoluble protein, increased rather than decreased the nutritive value of the remainder of the potato.

The results of amino-acid analyses of tuberin and protein-free juice do not provide an explanation for the complementary action between these two fractions. From the results given in Table 1 it is clear that any replacement of tuberin nitrogen by nitrogen from protein-free juice would lead to a reduction in the amount of ten essential amino-acids present. Nor, unless some insoluble protein which could not account for more than 7–10% of the total potato nitrogen were present in potato pulp, and were exceptionally rich in these essential amino-acids, could the whole potato contain more of these than is contained in a corresponding amount of tuberin.

Two explanations of this conflicting series of findings are possible. (i) The feeding experiments with separated potato fractions may be vitiated by chemical changes taking place during separation of the fractions, thus making a fair comparison with the intact potato impossible. (ii) The potato may contain substances, other than proteins and amino-acids, which remain undetected by the usual chemical methods of examination, but which are capable of exerting a supplementary or sparing effect upon protein.

A recent paper by Rose, Oesterling & Womack (1948) contains a description of experiments in which growth of weanling rats was definitely stimulated by addition of 2 % glutamic acid to a diet in which the nitrogen, adequate in amount, was supplied as a mixture of the ten 'essential' amino-acids. It is therefore possible that some nutritional significance attaches to the relatively large amounts of glutamine present in the potato tuber. In the sample of King Edward potatoes, of which the analysis is given in Table 1, 11·4 % of the total nitrogen and 24 % of the non-protein nitrogen were present as glutamine, the corresponding values for the content of free amino-acids being 10·1 and 21·5 % respectively.

SUMMARY

1. In the variety of potato studied, King Edward grown in the Fenlands, the total nitrogen varied from 1·7–2 % on dry weight; the proportion present as protein varied from about 40 to 50 % and was slightly lowered when the potatoes were stored. In the expressed juice of the raw potatoes about 30 % of the nitrogen was protein nitrogen: of the non-protein nitrogen about one-fifth was present in free amino-acids, two-fifths in amides and two-fifths in nitrogenous bases. After extraction of the residual pulp with salt solution and alkali, 7–10 % of the total nitrogen remained unextracted.

2. At least two soluble proteins were detected in the press juice after deposition of the particles of the starch; one (α-globulin) precipitated from the diluted sap on standing at pH 4, the other (β-globulin) on boiling the filtrate therefrom. In one specimen of freshly dug potatoes the ratio of α- to β-globulin was about 1/2: in a sample of stored potatoes it was about 2/1.

3. Both the nutritive value, judged by capacity to support growth in weanling rats, of the nitrogen in the whole steamed potato and the economy of its utilization, were less than those of the nitrogen in potatoes deprived of the skin and still lower than those of the nitrogen in peeled potatoes from which a layer of 2 mm. thickness had been removed.

4. The growth-promoting value of the potato nitrogen was usually found to be diminished after storage; the extent of deterioration depended on the conditions, being least after storage at 5°. The ratio of protein to non-protein nitrogen was also lowered after storage.

5. On the basis of nitrogen content, the nutritive value of tuberin prepared fron the sap by heating at 80° at pH 4·0 was not superior to that of the mixture of protein and non-protein nitrogenous material in the whole potato. The non-protein nitrogen in the press juice, after removal of the tuberin, was unable to support growth, but when combined with tuberin in the proportions (56/44) in which non-protein and protein nitrogen existed in the whole potato, the nutritive value was raised to a level approximating to that of tuberin, although still inferior to that of the nitrogenous mixture *in situ* in the intact tuber. This result shows the existence of a complementary nutritive action between the protein and non-protein nitrogen in the potato.

6. The coefficient of (apparent) digestibility of the nitrogen was about 76 in the intact potato, about 65 for tuberin, 83 for the press juice and about 69 for the artificially reconstituted potato. The relatively low digestibility of the nitrogen in the separated protein, and of that in the mixture of this with the pulp, may partly account for the inferior nutritive value of the artificially reconstructed potato compared with the original it was designed to simulate.

7. The supplementary nutritive effect between the tuberin and the protein-free fraction of the press juice could not be explained in terms of their amino-acid contents. Nor, unless the potato contains an insoluble protein richer in essential amino-acids than is tuberin, should the whole potato furnish these amino-acids as abundantly as can tuberin. The somewhat inferior value of tuberin, compared with that of the mixture of nitrogenous substances in the intact tuber, may possibly be due to chemical degradation during its separation.

We desire to thank Sir Charles Martin, F.R.S., for his encouragement and helpful criticism during the course of this work and for his hospitality at Roebuck House, Chesterton, Cambridge, to which, at his invitation, the Division of Nutrition of the Lister Institute was evacuated during the war years. We are also indebted to Mrs M. E. M. Cutting for help in carrying out Exp. 3 and to Mr G. W. Flynn for assistance in the care of the experimental animals.

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The Pectic Enzymes of the Mould *Byssochlamys fulva*

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The fungus *Byssochlamys fulva* was discovered by Olliver & Smith (1933), and its action on pectic substances first studied by Olliver & Rendle (1934). Hirst & McMaster (1932–3); Hull (1933–4, 1934–5, 1939) and Gillespy (1936–7, 1939, 1940, 1946) have described its characteristics, while its metabolic products have been investigated by Raistrick & Smith (1933). It derives its economic importance from three characteristics, two of which are unusual or even unique: (*a*) its ascospores have an abnormally high heat resistance and can readily withstand the normal processes used in bottling fruit, though only a small proportion survive the higher temperatures used in canning, (*b*) it can grow under conditions of greatly reduced oxygen tension, e.g. in canned and bottled fruit, and (*c*) it secretes pectic enzymes which can cause disintegration of the fruit tissue. Ascospores of *B. fulva* are thus liable to survive the usual bottling processes and cause more or less complete breakdown of the fruit.

The present work is an attempt to identify the particular pectic enzymes involved. The nomenclature used in this paper is in conformity with current views on the structure of pectin (Hirst & Jones, 1946), which support the classification of pectic enzymes proposed by Kertesz (1936): (1) protopectinase (or pectosase) which converts protopectin into soluble pectin; (2) pectin methoxylase (or pectase) which converts pectin into pectic acid and methanol; and (3) pectinase (probably a group of enzymes) which causes a reduction in the molecular size of the pectin and leads ultimately to D-galacturonic acid. The group contains at least a disaggregating enzyme and polygalacturonase.

EXPERIMENTAL

Enzymes. Three sources were used: (1) bottled fruit which showed pronounced disintegration due to infection with *B. fulva*, the fruit juice being separated from the disintegrated fruit tissues by repeated filtration through cotton wool; (2) Czapek-Dox solution on which a pure culture of the mould had been grown; (3) extracts of the fungal mycelium collected from various types of culture solution. More concentrated enzyme preparations were obtained from each of these sources by the addition of ethanol (3 vol. to 1 vol. of solution) and separation of the flocculent precipitate in a centrifuge. The solid product was freed from ethanol by drying over $CaCl_2$ in an evacuated desiccator and then dissolved in a small volume of water. The solution was kept sterile by storage at low temperature over $CHCl_3$.

Substrates. Potato disks of standard dimensions (1·2 cm. diam., 0·5 mm. thick) prepared on a simple hand microtome were used for the protopectinase tests. Pectin solutions (prepared from citrus pectin, 100 grade, British Drug Houses Ltd.) and polygalacturonide methyl ester, prepared by the action of methanolic HCl on pectin (Morell, Baur & Link, 1934) were used for the pectin methoxylase and pectinase tests. Pectic acid, prepared by demethoxylating pectin with pectin methoxylase from tomatoes, was also used for some of the pectinase tests.

Methods employed for enzyme detection. (*a*) Protopectinase was detected by its macerating action on thin disks of potato tuber at 37°. The disks were periodically removed from the enzyme solution and any maceration observed by gently pulling them with blunt-ended forceps. The results were placed on a semi-quantitative basis by using a scale of softening ranging from 'just detectable' (+) to 'complete disintegration' (++++). (*b*) Pectin methoxylase activity was examined by Kertesz's (1937) method in which the carboxylic acid groups set free by the action of the enzyme are titrated with dilute alkali, using methyl red as indicator. (*c*) Pectinase activity is difficult to assess because of the complexity of the reactions involved. Periodic determinations of the viscosity and of the reducing power of pectin solutions on treatment with the enzyme preparation were chosen as measuring respectively the concentrations of unchanged substrate and the final decomposition product, D-galacturonic acid.

Blank experiments with heat-inactivated enzyme solutions were included in all tests.

RESULTS

Protopectinase. Protopectinase activity was detected in four out of seven cultures of *B. fulva* in Czapek-Dox medium all containing 5 % glucose, and in some cases, 0·05 % asparagine. The most active culture gave + and + + ratings in the potato disk test in 3 and 18 hr. respectively. Two of those inactive were anaerobic cultures with submerged flocculent mycelia, in contrast to the thick white surface mycelia observed in aerobic cultures at pH 3–4. The single inactive aerobic culture was grown at pH 5 and formed a partly submerged mycelium similar to the anaerobic cultures. There was some indication that the addition of asparagine to the culture medium enhanced the production of protopectinase.

Protopectinase activity was detected in five out of six specimens of bottled fruit (plums, damsons, gooseberries) showing partial or complete disintegration of the fruit tissue. The most active specimen gave a potato disk rating of + + in 6 hr., the remaining four showing + + + in 24 hr.

Activity tests over the pH range 2–8 were made with filtered fruit juice, the pH of the juice being adjusted with N-acid or alkali. Maximum activity was observed at pH 4–5 (cf. Davison & Willaman, 1927; Brown 1915, who gave pH 5 and 6 respectively).

Experiments on the thermal inactivation of the enzyme at pH 4 are shown in Table 1. The results indicate that the enzyme is completely inactivated in a medium of pH 4 by exposure to a temperature of 65° for 5–10 min.

Table 1. *Effect of heat on protopectinase of* Byssochlamys fulva

	Time of heating (min.)			
	5	10	15	20
Temp. (°)	Activity rating (see test)			
50	+ +	+ +	+ +	+ +
55	+ +	+ +	+ +	+ +
60	+ +	+ +	+ +	+
65	±	−	−	−
70	−	−	−	−

Pectin methoxylase. This enzyme was consistently absent from *B. fulva* culture media, from extracts of the fungal mycelium, and from the juice of infected bottles of fruit. The specimens tested were the same as those used in the protopectinase tests.

Control experiments with tomato juice and the commercial pectic enzyme preparation known as 'Pectinol 10 M,' using 1 % pectin solutions as substrate, showed that Kertesz's (1937) method is only suitable for the qualitative demonstration of pectin methoxylase in an enzyme system. Quantitative tests are made difficult by an apparent dependence of the activity of the enzyme sample (in 'Kertesz units'/ml.), on the size of the sample and the duration of the test. In a typical experiment a 1 % pectin solution (40 ml.), which had been adjusted to pH 6·5 with 0·1 N-sodium hydroxide using methyl red as indicator, was treated with 1 ml. of a 'Pectinol 10 M' solution containing 0·2 mg. of the enzyme; acid was formed in the solution in 2·5 hr. at 15°. In similar experiments, using *B. fulva* enzyme preparations in various concentrations (1–5 ml. of the concentrated solution in 40 ml. of 1 % pectin solution), no acid was produced, even after 5 days. Further experiments at 30° again pointed to the absence of pectin methoxylase activity in the *B. fulva* enzyme system, whereas at this temperature 'Pectinol 10 M', in the same concentration as before, produced acid within 1 hr.

Independent pH measurements using a Cambridge pH meter confirmed the failure of all the *B. fulva* enzyme preparations to produce acid from a pectin substrate.

Pectinase complex. Pectin solutions which were treated with *B. fulva* enzyme preparations at 25 and 37° showed a rapid initial fall in viscosity, followed by a slower fall to a constant value (see Fig. 1).

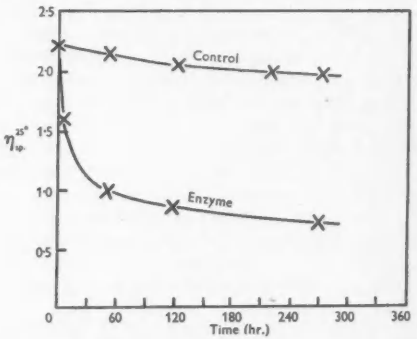

Fig. 1. Effect of *B. fulva* enzymes on the viscosity of 1 % pectin solution at 25°.

Simultaneous determinations of the reducing power by the alkaline iodide method (Willstätter & Schudel, 1918; Ingles & Israel, 1948) indicated that only a minute increase took place which was of the same order as the experimental error of the estimation (0·05 ml. of 0·1 N-iodine). Typical results are shown in Table 2.

The results obtained over the pH range 3–5 were quantitatively similar and an optimum pH value was not apparent.

Control experiments with 'Pectinol 10 M' at 37° indicated that the reducing power of 0·4 % pectin solution did not increase until the specific viscosity of the solution had fallen to a value of approxi-

mately 0·2. Further tests were thus made with a *B. fulva* enzyme preparation in which the pectin solutions were held for long periods at 37° after the viscosity had reached a constant value ($\eta_{sp.}^{25°} = 0·12$). Even 1000 hr. after the viscosity of the pectin solution had become constant, the increase in reducing power remained negligible. On the other hand, 0·4 % pectin solution (40 ml.) which had been treated with 0·2 mg. of 'Pectinol 10 M' at 37° rapidly gave an increase in reducing power equivalent to 0·29 ml. of 0·1 N-iodine/2 ml. of pectin solution.

Table 2. *Effect of* Byssochlamys fulva *enzymes on the viscosity and reducing power of pectin*

Pectin (%)	Mixture	Temp. (°)	Viscosity ($\eta_{sp.}^{25°}$) Initial	Viscosity ($\eta_{sp.}^{25°}$) Final	Reducing power of 2 ml. mixture (ml. 0·1 N-I_2) Initial	Reducing power of 2 ml. mixture (ml. 0·1 N-I_2) Final
1·0	Test	25	2·22	0·70	0·13	0·15
1·0	Blank	25	2·22	1·99	0·10	0·13
0·4	Test	37	1·31	0·12	0·33	0·38
0·4	Blank	37	1·31	1·02	0·34	0·39

The material recovered by precipitation with ethanol from the *B. fulva* test and from blank solutions after the viscosities had become constant showed markedly different physical properties. The precipitate from the test solution was non-gelatinous and flocculent, whereas that from the blank solution was gelatinous and voluminous, resembling undegraded pectin precipitated under the same conditions.

A 1 % polygalacturonide methyl ester solution ($\eta_{sp.}^{25°} = 0·27$) was treated with an extract of *B. fulva* mycelium which rapidly disaggregated pectin. The viscosity of the solution did not fall in 300 hr. and the reducing power of the polyester was similarly unaffected.

In view of the fact that Jansen & MacDonnell (1945) have stated that only de-esterified pectic substances are attacked by polygalacturonase, a suspension of pectic acid in water was treated with the *B. fulva* enzymes. Failure to obtain any increase in reducing power further confirmed the absence of polygalacturonase from the *B. fulva* enzyme system.

DISCUSSION

The present results show that *B. fulva* produces an enzyme system possessing protopectinase and pectinase activity. Protopectinase activity is found in many fungi and bacteria (Branfoot, 1929) and it has long been recognized that the macerating action of many plant pathogens is due to the production of protopectinase and the consequent destruction of the middle lamella. Protopectin, the substrate for this enzyme, is ill defined so that work on this enzyme has been mainly qualitative.

Although little attention has been paid to the individual components of the complex, the pectinase group of enzymes appears to contain at least polygalacturonase and a disaggregating enzyme. Many workers have obtained pectinase preparations with very marked polygalacturonase activity; thus Ehrlich (1933, 1935), Mottern & Cole (1939), and Manville, Reithel & Yamada (1939) have broken down pectin completely into D-galacturonic acid. On the other hand, Kertesz (1939) has observed that many pectinase preparations from various Penicillia bring about the development of only a small fraction of the maximum possible reducing power from pectin. This variation in activity may be due to the existence of two or more polygalacturonases, acting on pectic substances of various molecular sizes, so that all the enzymes are necessary to bring about the complete sequence of reactions required to produce D-galacturonic acid. Kertesz (1939) has suggested that two different types of enzyme are involved in pectinase activity; the first disaggregating the pectin molecule, with a consequent fall in viscosity, and the second attacking the product so formed, opening the glycosidic bonds between the uronic acid residues with a consequent increase in reducing power.

The results of the present investigation indicate that a very large reduction in the molecular size of pectin is brought about by the enzyme system of *B. fulva*, without any increase in reducing power, and therefore without any fission of glycosidic bonds. This result is an extreme case of the phenomenon noted by Kertesz (1939) amongst the pectinases of various Penicillia. It is considered that the results provide confirmatory evidence for Kertesz's view that the large molecular size of pectin is mainly due to the existence of aggregates, consisting of relatively small polyuronic acid units joined by linkages which are not glycosidic but possibly of the nature of hydrogen bonds.

SUMMARY

1. The enzyme system produced by the mould *Byssochlamys fulva* has been examined. Evidence has been found for the presence of protopectinase and a disaggregating enzyme which reduces the molecular size of pectin without the production of galacturonic acid.

2. The production by a fungus of a pectinase type of enzyme which is free from pectin methoxylase has not been reported hitherto.

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Studies on Suramin (Antrypol: Bayer 205)

7. FURTHER OBSERVATIONS ON THE COMBINATION OF THE DRUG WITH PROTEINS

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Little is known about the metabolic fate of injected suramin, the trypanocidal drug which is retained in the body for several weeks or even months after its injection. Even before satisfactory methods for the determination of suramin became available, however, it was suggested that the injected drug combines with the body proteins (for a review of the early literature see Findlay, 1939), and this view received support from the observation that the addition of suramin effects marked changes in some of the properties of certain proteins; for example, the serum proteins are protected against heat coagulation (Collier, 1926, 1927) and against precipitation by tannin, mercuric chloride and certain other reagents (Jirovec & Koçian, 1930; Kocian, 1936), the isoelectric points of some immune substances are altered (Klopstock, 1932), and the drug inhibits a few enzymes, e.g. trypsin (Beilinsohn, 1929), fumarase (Quastel, 1931) and hyaluronidase (Beiler & Martin, 1948).

Quantitative measurement of the amount of suramin which combines under varying conditions with the plasma proteins of animals injected with the drug, or in experiments *in vitro* with various proteins, became possible when a satisfactory chemical method became available for the determination of the drug (Dangerfield, Gaunt & Wormall, 1938). Using this method it was conclusively shown that suramin can combine with many different proteins (serum globulin, fibrinogen, casein, gelatin) under physiological conditions of pH and temperature (Boursnell & Wormall, 1939; Dewey & Wormall, 1946).

At present we are investigating the action of suramin on enzymes. It was thought, therefore, that a study of the conditions under which the drug combines with proteins, and a further investigation of the nature of the linkages concerned, might throw some light on the specific inhibitory effect which the drug exerts on some enzyme systems. It was also hoped that useful information might be obtained about the mechanism whereby suramin inactivates complement and the blood-clotting system.

The present paper describes a study of (i) the stability of the linkage between the drug and various proteins, (ii) the influence of pH on the combination,

(iii) the failure of suramin to combine, *in vivo* and *in vitro*, with the proteins of the intact red blood cell, (iv) the fate of intravenously injected suramin-protein complexes and (v) the protein groups which might be concerned with the binding of suramin.

METHODS AND MATERIALS

Suramin determinations. These were carried out as described previously (Dangerfield *et al.* 1938; Boursnell, Dangerfield & Wormall, 1939; Dewey & Wormall, 1948). The values for rabbit plasma have been corrected for the small amount of amine precursor (equivalent to about 1·0 mg. suramin/100 ml.) present in normal plasma. A similar correction has been made for the precipitated suramin-protein complexes, but this blank value is usually negligible (equivalent to 0·01–0·02 g. suramin/16 g. protein N).

Total N determinations were carried out on most of the precipitated suramin-protein complexes, and the suramin contents of the complexes (except those referred to in Table 1) are given as g. of suramin/100 g. of the complex, allowance being made for the N content of suramin (5·88%). The N content of the untreated proteins was assumed to be 16% for the mixed serum proteins and 15·76% for crystalline egg albumin (Chibnall, Rees & Williams, 1943). This method of recording the results was adopted since the methanol-precipitated complexes contained appreciable amounts of inorganic material.

Injections. The solutions of suramin or suramin-protein complexes, in 0·9% (w/v) NaCl, were injected into the marginal ear veins of rabbits, and blood samples were taken from the ear not used for the injection and collected in 'oxalated' tubes. The plasma was then separated as quickly as possible.

Amino-N determinations. These were made by titration from pH 7 to 9, with a further titration back to pH 9 after the addition of excess of neutralized 20% formaldehyde solution. (For full details see Boursnell, Francis & Wormall, 1946).

Buffer solutions. The 'Universal' buffer (Baird and Tatlock (London), Ltd.) of Johnson & Lindsey (1939) was used for the range pH 3–9, and the sodium acetate-HCl buffer of Walpole (cf. Vogel, 1944) for pH 1. For some experiments a 0·75 M-$NaHCO_3$-H_2CO_3 buffer of pH 7·5 was used.

Egg albumin. This was thrice crystallized hen ovalbumin.

RESULTS

The precipitation of suramin-protein complexes by dilute acid

It was found that mixtures of suramin and serum gave heavy precipitates when the solutions were made slightly acid, the pH of maximum precipitation varying with the protein used and the amount of suramin added. These precipitated complexes contained much suramin, and it was decided to determine whether repeated solution and precipitation of these complexes would alter their suramin content. In a typical experiment the drug-protein complex was precipitated from a mixture of suramin and human serum by adjusting the pH to about 4·0, followed by solution in 0·9% NaCl at pH 7·5, and a repetition of the precipitation a further seven times. Analysis of the precipitates, some washed with 0·9% NaCl and some washed with methanol, showed that there was very marked consistency in the suramin content of the complexes during this serial precipitation, even up to the eighth precipitation (Table 1). The methanol-washed precipitates contained slightly less suramin than did those washed with NaCl, but in this case also there was no fall in the suramin content of the complex during five precipitations.

Table 1. *Suramin content of acetic acid-precipitated suramin-protein complexes from a mixture of suramin and human serum*

(Suramin, 1·0 g. dissolved in 5·0 ml. of 0·9% NaCl, was added to 150 ml. of human serum and the mixture kept for 1 hr. at room temperature. The suramin-protein complex was then precipitated by the addition of N-HCl and finally 2N-acetic acid to give maximum precipitation (pH about 4·0). Suramin determinations were made on part of the precipitate and the rest was dissolved in 0·9% NaCl with the addition of a little 0·75M-Na_2CO_3-H_2CO_3 buffer of pH 7·5. Precipitation and solution were repeated a further seven times and suramin determinations were made on samples of the precipitates washed (*a*) twice with 0·9% NaCl, or (*b*) twice with methanol).

No. of precipitations	Suramin content of the precipitates (%)	
	NaCl-washed	Methanol-extracted
1	13·4	11·8
2	12·4	11·9
3	13·5	12·4
4	13·8	11·8
5	13·3	13·1
8	12·8	—

In other experiments egg-albumin solutions were treated with varying amounts of suramin, the concentration of the drug in the mixture ranging from 0·21 to 1·67% (i.e. $1·5–11·7 \times 10^{-3}$ M). The drug-protein complexes were precipitated by adding acetic acid, and after five precipitations contained 10·74–17·54 g. of suramin/100 g. of the drug-protein complex (Table 2). The amount of drug bound by the protein in the mixture with the lowest concentration of suramin used in this experiment was surprisingly high; it was 10·74 g. suramin/100 g. of the drug-protein complex, this being equivalent to the combination of 12·0 g. of suramin with 100 g. of protein, or 3·7 suramin groups/molecule of egg albumin, assuming a molecular weight of 44,000 for this protein.

The product obtained from the mixture with the highest concentration of suramin used in this experiment contained 17·54 g. of suramin/100 g. of the drug-protein complex. This is equivalent to the

combination of about 21 g. of suramin with 100 g. of egg albumin, or the average introduction of 6·5 suramin groups into each protein molecule.

Table 2. *Combination of egg albumin with varying amounts of suramin*

(Varying amounts of a 10% (w/v) solution of suramin were added to samples of a 2·36% solution of crystalline egg albumin and the mixtures kept at about 14° for 30 min. The suramin-protein complexes were then repeatedly precipitated by the addition of 5N-acetic acid, and dissolved in water at about pH 7·5; five precipitations in all.)

Egg albumin solution (ml.)	Suramin solution (ml.)	Water (ml.)	Suramin content of precipitated complex (g./g. total N)	Suramin content of precipitated complex (g./100 g. suramin-protein)
20	0·5	3·5	0·73	10·74
20	2·0	2·0	1·18	16·65
20	4·0	0	1·25	17·54

Influence of pH *on the combination of suramin with proteins*

Suramin undoubtedly combines firmly with serum and other proteins at pH 7·5, but the amount of suramin combining at that pH is not necessarily the same as that present in the suramin-protein complexes precipitated from the same solution by the addition of acid. Experiments were therefore made to determine the amount of suramin combining with proteins at different pH's. The drug-protein complexes were precipitated with methanol, without materially altering the pH of the mixture, and the precipitates were washed with methanol, in which free suramin is very soluble.

N-HCl or 0·1N-NaOH was added to rabbit serum (2 ml.) to give approximately the pH required. Buffer solution was added, together with 0·9% NaCl to give a total volume of 5·5 ml. The suramin solution (1·0 ml. of a 3% (w/v) solution in 0·9% NaCl) was then added to each tube, and the mixture was shaken and kept at room temperature for 1 hr. with occasional shaking. Methanol (26 ml.) was then added to each mixture, and the centrifuged precipitates were washed four times with methanol (7 ml. each time), and dried *in vacuo* over $CaCl_2$.

The results of this experiment (Table 3) showed that under these conditions the serum proteins combined at pH 1 or 3 with about twice as much suramin as they did at pH 5. At pH 7 the amount of combined suramin was only about one-quarter, and at pH 9 about one-fifteenth, that combined at pH 5. A similar experiment was carried out with egg albumin over the same pH range, but with additional mixtures at pH's 6 and 8.

The solution of crystalline egg albumin (2 ml. of 5·8% w/v) was brought to the required pH and mixed with buffer, 0·9% NaCl and suramin solution as in the above experiment with rabbit serum proteins. The suramin-protein complexes were precipitated and washed with methanol and were then dried and analysed.

The results (Table 3) were similar to those obtained with the serum proteins; they showed that much more suramin combines with protein in the more acid solutions used in these experiments.

Table 3. *The influence of* pH *on the combination of suramin with normal rabbit serum proteins and with egg albumin*

(See the text for experimental details. Each experiment was carried out in duplicate and the two sets of results are recorded below.)

pH	Suramin content (g./100 g. suramin-protein complex) of the methanol-precipitated complexes of suramin and: Rabbit serum proteins	Egg albumin
1	22·9, 22·7	20·95, 20·95
3	22·6, 22·5	19·7, 18·7
5	11·9, 10·6	4·6, 5·2
6		0·63, 0·76
7	2·8, 2·5	0·49, 0·59
8		0·28, 0·34
9	0·80, 0·64	0·31, 0·23

Combination of suramin with the proteins of red blood cells

Earlier investigations (Dewey & Wormall, 1948) have shown that suramin added to whole blood, or to suspensions of red blood cells, does not pass into the red cell. This might be due to inability of the drug to pass through the membrane, or to an inability of suramin to combine with haemoglobin and the stroma protein.

Although suramin did not enter the red cell in these *in vitro* experiments it does not follow that the same will hold true *in vivo*. Experiments have therefore been made to determine whether the drug enters the red cells after it has been intravenously injected into rabbits. Estimations were made on the separated red cells after they had been washed with 0·9% NaCl, and also on the unwashed cells (to exclude the possibility of loss of suramin by the cells during the washing process). In all these experiments, with plasma-suramin values up to 69 mg./100 ml., no significant amount of the drug was found in the red cells; the very small amount occasionally found in the unwashed red cells was not more than could be accounted for by the presence of a small amount of suramin-containing plasma in the cell fractions.

The second possibility mentioned above was examined by determining the capacity of haemolysed red cells to combine with suramin. In one group of experiments, the results of some of which are given in Table 4, the red cells of normal rabbit blood were washed with 0·9% NaCl, haemolysed

Table 4. *Combination of suramin with the plasma and cell proteins of normal rabbit blood*

(The suramin solution (2·8 of 10% (w/v) in 0·9% NaCl solution) was added to the mixture of plasma and 0·9% NaCl, or to the haemolysed red cell solution. Each mixture was kept at room temperature for 15 min. with occasional shaking; it was then treated with an equal volume of 0·9% NaCl solution and the protein-suramin complex precipitated by the addition of 2 N-acetic acid. The precipitate was dissolved in 3 ml. of 0·9% NaCl solution with the aid of sufficient 2 N-Na_2CO_3 to give pH about 7·5, and the precipitation with acetic acid was repeated a further five times.)

Exp. no.		Plasma or haemolysed cells* (ml.)	0·9% NaCl (ml.)	Suramin solution added (ml.)	Suramin solution added (mg. suramin)	Suramin content of precipitated suramin-protein complex (g./g. total N)	Suramin content of precipitated suramin-protein complex (g./100 g. complex)
1	Plasma	1·5	1·5	0·5	14	0·73	10·9
		1·5	1·5	1·0	28	0·96	14·0
	Haemolysed cells	3·0	0	0·5	14	1·13	16·2
		3·0	0	1·0	28	1·47	20·5
2	Plasma	3·0	5·0	1·0	100	1·88	25·3
		3·0	5·0	2·0	200	2·03	26·9
	Haemolysed cells	8·0	0	1·0	100	1·72	23·4
		8·0	0	2·0	200	1·89	25·4

* Centrifuged cells from 1 vol. of oxalated rabbit blood were washed well with 0·9% NaCl and then haemolysed with 4·5 vol. of distilled water.

with water, and then mixed with a suramin solution, parallel experiments being made with the blood plasma. The mixtures of suramin and haemolysed red cells gave heavy precipitates when dilute acid was added and these complexes, reprecipitated a further five times, had a suramin content which was of the same order as that of the complexes separated similarly from the corresponding suramin-plasma mixtures. For example, in Exp. 2, where the plasma and haemolysed cells were diluted so that each solution contained the same amount (0·38%) of protein N before the addition of the suramin solution, there was a very close similarity between the suramin contents of the complexes from suramin-plasma mixtures and those from mixtures of suramin and haemolysed red cells.

The retention of injected suramin-protein complexes

Experiments have been made to determine whether intravenously injected suramin-protein complex is retained longer in the blood of a rabbit than is an equivalent amount of the free drug. In one experiment the drug was attached to crystalline egg albumin, and in the other rabbit serum proteins were used to form the complex, in the hope that this would

Table 5. *The suramin content of the blood plasma of rabbits following the intravenous injection of (a) suramin and (b) suramin-protein complexes*

(For experimental details see the text.)

Exp. no.	Material injected	Suramin injected (mg./kg.)	Suramin content of plasma (mg./100 ml.) after 17 min.	45 min.	3 hr.	24 hr.	48 hr.	7 days	14 days
1	Suramin	14·2		20·0	12·0	4·2	3·2	1·6	
		23·3*	33·4		14·8	7·5	4·8	1·8	
	Suramin-rabbit serum protein complex	13·05		21·5	8·1	1·8	1·5	1·0	
		22·0*	46·3		26·6	7·9	5·5	3·0	
2	Suramin	21·9	46·5		19·8	6·4	4·4	2·4	1·6
			39·2		15·2	5·9	4·0	1·9	1·6
			42·0		16·4	6·6	4·3	2·3	2·0
	Suramin-egg albumin complex	21·8 (*a*)†	41·3		15·8	7·0	5·6	1·6	1·7
			37·2		15·5	5·9	4·1	1·6	1·1
		22·1 (*b*)†	37·0		12·8	5·7	3·5	1·9	1·7
			36·6		15·3	6·2	3·4	2·1	1·6

* Given in two injections, with an interval of 36 min. between them. The times of bleedings were reckoned from the second injection.

† (*a*) and (*b*). After it had been kept at 5° for a few days, the solution of suramin-egg albumin complex prepared for this experiment contained a very small amount of solid matter. Two rabbits were injected with the uncentrifuged solution (*b*) and two with the clear centrifuged solution (*a*).

be less 'foreign' than the egg albumin complex and therefore less liable to suffer rapid breakdown in the rabbit.

Exp. 1. A suramin solution (300 mg. in 10 ml. of 0·9% NaCl) was added to a mixture of rabbit serum (15 ml.) and 0·9% NaCl (15 ml.), the mixture kept at about 13° for 30 min. and the drug-protein complexes precipitated by the addition of 5N-acetic acid to give maximum precipitation. After eight further precipitations with acid, the complex was dissolved in 0·9% NaCl (15 ml.) at pH 7·0 and filtered through a Berkefeld filter. This solution, containing 0·88 g. of suramin and 3·05 g. of protein/100 ml., was injected into rabbits as follows: one rabbit (wt. 2·7 kg.) received 4·0 ml., and another rabbit (wt. 3·0 kg.) received 4·5 ml. followed by a further injection (3·0 ml.) 36 min. later. Two control rabbits were injected under the same conditions with a solution containing 0·88 g. of suramin/100 ml. of 0·9% NaCl. Blood samples (about 7 ml.) were taken from the rabbits at intervals for plasma-suramin determinations.

Exp. 2. Suramin (1·5 g. in 15 ml. of water) was added to a solution of crystalline egg albumin (150 ml. containing 3·54 g. of protein) and the drug-protein complex was precipitated by acid and purified by four further precipitations, as in Exp. 1. The final solution (containing 1·26 g. of suramin, 6·59 g. of protein, 0·9 g. of NaCl and 0·04 g. of merthiolate/100 ml.) was injected into four rabbits (one injection each), and three control rabbits received corresponding injections of free suramin.

The results of these experiments (Table 5) showed that the injected suramin-protein complex was not retained longer in the plasma than was an equivalent amount of the free drug. Individual rabbits occasionally show appreciable deviation from the average with regard to their retention of the injected drug or drug-protein complex, but in no case did any rabbit injected with the complex show any abnormally long retention of the suramin.

The combination of suramin with proteins treated with di-2-chloroethylsulphone

The combination of suramin with proteins might be due to a reaction between the sulphonic acid groups of the drug and basic groups of the protein. Tests were therefore made to determine whether the blocking of the free amino groups of the protein reduces its capacity to combine with suramin. Blocking of practically all the amino groups of gelatin can readily be effected at pH 8 and 30° by mustard-gas sulphone, di-2-chloroethylsulphone (Banks, Boursnell, Francis, Hopwood & Wormall, 1946), and the ability of the sulphone-treated proteins (gelatin and the serum proteins) to combine with suramin was, therefore, compared with that of the untreated proteins.

Exp. 1. Gelatin (1·0 g.) was dissolved in hot water (60 ml.), the solution cooled a little and 0·75M-$NaHCO_3$ (40 ml.) added. The mixture was treated with di-2-chloroethylsulphone (0·5 g.), stirred at 37° for 4 hr. and then kept at 4° overnight. Next day a further 0·5 g. of the sulphone was added and the mixture stirred at 37° for a further 8·5 hr. The solution was then diluted with an equal volume of water and dialysed against distilled water for 24 hr. at 4°. Total N and amino N (formaldehyde) determinations on this solution and on a dialysed 0·5% gelatin solution showed that the sulphone had blocked at least 95% of the free amino groups of the gelatin.

The dialysed gelatin and sulphone-gelatin solutions (each containing 0·45 % (w/v) of protein) were each treated with buffer solution and suramin (see Table 6 for details) and the mixtures kept at 18° for 1 hr. Each solution (36 ml.) was then poured into ice-cold methanol (220 ml.) and the precipitated complexes were centrifuged and washed three times with ice-cold methanol (25 ml. each time). Mixtures *C* and *D* required the addition of a few drops of glacial acetic acid before the drug-protein complex could be precipitated by the methanol, and the methanol used for washing these precipitates was similarly acidified. The dried drug-protein complexes were dissolved in warm water (with the aid of a few drops of N-NaOH in the case of *C* and *D*) and total N and suramin determinations were made on samples of these solutions.

Exp. 2. Di-2-chloroethylsulphone (1·0 g.) was added to a mixture of rabbit serum (24 ml.) and 0·5M-$NaHCO_3$ (24 ml.) and the mixture stirred at 37° for 6 hr., N-NaOH being added when necessary to maintain the pH between 7·5 and 8·5; after 2 days at 4°, the solution was again stirred at 37° for 6 hr. after the addition of more sulphone (1·0 g.). The mixed 'sulphone-proteins' were precipitated by adding dilute acetic acid, dissolved at pH 7–7·5 and purified by three precipitations with N-HCl. The final solution (20 ml.) contained 4·86% protein, 0·9% NaCl and 0·02% merthiolate; total N and amino N determinations on this solution and on dialysed rabbit serum showed that 72% of the free amino groups of the serum proteins had been blocked by treatment with the sulphone.

Samples of the diluted solution of sulphone-serum proteins were treated with varying amounts of suramin, as recorded in Table 7, and the mixtures were kept at 15° for 30 min.; the drug-protein complexes were precipitated by addition of 5N-acetic acid and purified by a further four precipitations. The final precipitates were dissolved in 0·9% NaCl with adjustment of the pH to 7·0, and total N and suramin determinations were made on these solutions.

The results show that mustard gas sulphone-treated gelatin combines with more suramin than does an equivalent amount of gelatin under similar conditions (Table 6). This increase may be partly due to the fact that these drug-protein complexes gave maximum precipitation at different pH's, but several other factors may also be involved. A similar increase in the capacity of the protein to react with mustard gas occurs when serum globulin is treated with sufficient phenyl *iso*cyanate to block most of its free amino groups (Banks *et al.* 1946).

In other experiments it was found that the treatment of rabbit serum proteins with mustard-gas sulphone (resulting in the blocking of about three-quarters of the free amino groups of the proteins) did not reduce the capacity of the proteins to combine with suramin (Table 7).

Table 6. *Combination of suramin with gelatin and di-2-chloroethylsulphone-treated gelatin*

(For experimental details see the text.)

Protein		Vol. of protein solution (ml.)	$NaHCO_3$-H_2CO_3 buffer solution (pH 7·5) (ml.)	Suramin (mg. dissolved in 1 or 2 ml. of water)	Suramin content of the methanol-precipitated complexes (g./g. of total N)	Suramin content of the methanol-precipitated complexes (g./100 g. complex*)
Gelatin	*A*	33	2	20	0·17	2·99
	B	33	2	40	0·26	4·54
Sulphone-treated gelatin	*C*	33	2	20	0·22	3·69
	D	33	2	40	0·78	12·32

* Assuming 18·0% of N in gelatin and 17·18% N in the di-2-chloroethylsulphone-treated gelatin.

Table 7. *Combination of suramin with di-2-chloroethylsulphone-treated rabbit serum proteins*

(For the experimental details see the text.)

Sulphone-treated serum protein solution* (ml.)	Suramin (mg. dissolved in 0·2 ml. of water)	Suramin content of precipitated drug-protein complexes (g./g. total N)	Suramin content of precipitated drug-protein complexes (g./100 g. complex)†
2·0	2·5	0·87	12·01
2·0	5·0	1·59	20·71
2·0	10·0	1·85	23·61

* Containing 1% (w/v) of protein in 0·9% NaCl solution.
† Assuming 14·89% N in the sulphone-treated proteins (calculated on the basis of 16% of N in the mixed serum proteins and the reaction of the sulphone with 72% of the free amino groups of proteins).

DISCUSSION

The ability of suramin to combine with a variety of proteins under physiological conditions of pH and temperature is now well established, though the precise linkage by which the drug is firmly bound to the protein is still unknown. The reaction is rapid and although the mixtures of suramin and protein were usually allowed to stand for 30 min. or 1 hr. in the experiments described here, it is not suggested that this interval is necessary. Our enzyme inactivation experiments, a preliminary account of which has been given elsewhere (Town, Wills & Wormall, 1949), have shown that previous contact of drug and urease (and certain other enzymes) for 30 min. or so before addition of the substrate leads to an increased inhibition of the enzyme; but it is also true that the addition of suramin to solutions of serum proteins or egg albumin at pH 7·5 is followed by the immediate formation of drug-protein complexes. These complexes can be precipitated by methanol or ethanol at pH 7·5 or by acidification to pH 4–5. The acid-precipitated complex undoubtedly contains more suramin than does the complex present at pH 7·5, but there is no doubt that an appreciable amount of the drug is firmly fixed to proteins at pH 7·5. Combination of the drug with the body proteins can account for the long retention of the injected drug, and it seems probable that slow, continuous liberation of the free drug, possibly as a result of the hydrolysis of the protein of the complex, accounts for the relatively long protection against sleeping sickness which is afforded by one or more injections of suramin. The trypanocidal power of the drug-protein complex is not known, but we hope to study this in collaboration with Dr F. Hawking.

The experiments described here show that suramin can combine as readily with haemoglobin, and possibly other proteins of the red blood cell, as it does with the plasma proteins. The failure of injected suramin to combine with the red cell is, therefore, due to inability of the drug to pass through the red cell membrane, presumably because of the size and/or spatial configuration of the suramin molecule. Suramin will not pass through relatively permeable collodion membranes (Boursnell *et al.* 1939) and in general it does not diffuse through animal membranes. Hawking (1940), for example, found no suramin, or faint traces only, in the cerebrospinal fluid of patients injected with the drug, even when the plasma contained as much as 17·2 mg. of suramin/100 ml.

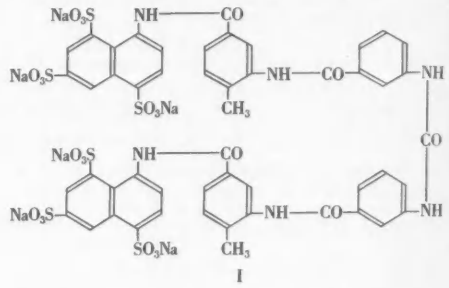
Our experiments with complexes of suramin and rabbit serum proteins (or egg albumin) have shown that retention of the drug in the blood following the intravenous injection of the preformed complex into a rabbit is not greater than that following the injection of an equivalent amount of uncombined suramin. These complexes will not, therefore, be of any special value for prophylactic purposes.

An appreciable amount of suramin combines with plasma proteins when the concentration of the drug is of the same order as that in the plasma after the injection of normal curative or preventive doses of 1 or 2 g. of suramin/70 kg. man. In some of our experiments, for example, we have found that the average plasma-suramin values of rabbits after a single intravenous injection of 28 mg. of suramin/kg. were as follows: 74 (2 min. after the injection), 69 (after 5 min.), 62 (after 10 min.) and 40 mg./100 ml. (after 40 min.). Protein-suramin complexes containing nearly 1% of the drug were separated by methanol precipitation at about pH 7·5 from the

plasma of these animals shortly after the injections and also from egg albumin solutions (or normal plasma) to which suramin had been added to the extent of 80–100 mg./100 ml. Even with much lower concentrations of suramin there is an appreciable amount firmly combined with the plasma proteins. Thus the plasma of rabbits 3 and 26 hr. after the injection of 28 mg. of suramin/kg. contained methanol-precipitable protein-drug complexes containing 0·24 and 0·08 % of suramin respectively.

The molecular weight of suramin is 1429, and the presence of a considerable amount of suramin in the complex does not require many suramin molecules/molecule of protein. In the experiments described in Table 2, for example, the complexes obtained contained 3·7–6·5 suramin groups/molecule of egg albumin (mol. wt. 44,000). The complexes obtained with rabbit serum proteins in a mixture containing 461·4 mg. of suramin/100 ml. at pH 7·0 (Table 3) contained the equivalent of about 2 mol. of the drug./mol. of serum protein (assuming an average mol. wt. of 100,000 for serum proteins). The complexes obtained in more acid solutions contain much larger amounts of suramin, and at pH 1 they corresponded to 21 mol. of the drug/mol. of rabbit serum proteins, and about 8·2 mol./mol. of egg albumin. However, at pH 7·5 and with suramin concentrations similar to those found in the plasma of rabbits shortly after the injection of 28 mg. of suramin/kg. (equivalent to a dose of 2 g. for a 70 kg. man), the drug-protein complexes contain much less suramin; the value corresponds usually to about 0·6 mol. of suramin/mol. of plasma protein.

Suramin (I) is a complex urea derivative containing three sulphonic acid radicals in each naphthylamine grouping. For several reasons it appeared probable that these naphthylaminesulphonic acid groups are responsible for the combination of the drug with proteins. We have found that acid-hydrolysed suramin, which contains *m*-aminobenzoic acid (II), *m*-amino-*p*-toluic acid (III) and 1-naphthylamine-4:6:8-trisulphonic acid (IV) (Lang, 1931), combines with proteins to produce acid-precipitable products. Tests with the individual amines showed that of these three amines only (IV) is capable of forming a complex with proteins; this complex is apparently much less stable than is the corresponding suramin-protein complex, since acid-hydrolysed suramin is not retained in the blood very long after its injection into rabbits (Dewey & Wormall, 1946). Furthermore, Spinks (1948) has shown that suramin analogues containing naphthalenepolysulphonic acids as end groups are generally similar to suramin with regard to their persistence in the blood after intravenous injection into rabbits; of the compounds of smaller molecular weight tested by this author, 1-naphthylamine-4:6:8-trisulphonic acid (IV) was fairly rapidly eliminated after injection but was retained longer than was (II) or (III). Spinks concludes that 'marked persistence is a property of polyamides of high molecular weight that contain naphthylaminepolysulphonic acids as end groups'. From the evidence presented by Spinks and by ourselves, it seems fairly certain that the ability of suramin to combine with proteins is determined by its sulphonic acid groups.



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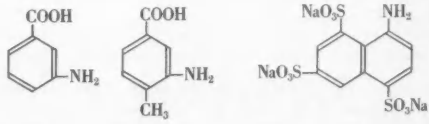

II III IV

Since the naphthylamine sulphonic acid groups of suramin appear to be concerned with this reaction, it seems most likely that they react with, or are bound by, basic groups of the protein. The results of our experiments suggest, however, that the free amino groups are not essential for the formation of suramin-protein complexes, and it would appear that other basic groups are involved. Mandel & Steudal (1926) showed that suramin readily combines with protamines and histones, and we are making a more extensive study of this combination, and of possible interactions between suramin and various amino-acid groupings, in the hope of gaining more information about the reaction between the drug and tissue proteins. A reaction with thiol groups can be excluded, since suramin does not appreciably reduce the thiol reaction of cysteine, reduced glutathione or heat-coagulated egg albumin. Furthermore, although the drug strongly inhibits urease at pH 5·0, it is not a general poison for thiol enzymes (Town *et al.* 1949; Wills & Wormall, 1949).

SUMMARY

1. A further study has been made of the combination of the trypanocidal drug suramin with a variety of proteins.

2. Suramin-protein complexes are usually readily precipitated by the addition of dilute acid, the pH

for optimum precipitation depending on the relative amounts of the drug and the protein. Repeated precipitation by acid followed by solution at pH 7·5 does not reduce the suramin content of the precipitated complexes.

3. Considerable amounts of suramin become firmly attached to proteins such as the serum proteins and crystalline egg albumin at pH 7·5, but much more is bound by the protein in acid solutions, particularly at pH's below 6.

4. The absence of suramin from the red cells of animals injected with the drug is due to the inability of the drug to pass through the cell membrane. Suramin readily combines with the proteins of haemolysed red cells.

5. Suramin injected intravenously as a complex with rabbit serum proteins, or with egg albumin, is not retained longer in the blood than is the injected free drug.

6. The blocking of the free amino groups of proteins by the action of di-2-chloroethylsulphone does not reduce the capacity of the proteins to combine with suramin.

7. Suramin does not react with the thiol groups of cysteine or proteins.

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The Phosphorus Compounds of Wheat Starch

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In a previous paper, Lampitt, Fuller & Goldenberg (1941) showed that the insoluble portion separating out when wheat starch retrogrades has a much higher phosphorus content than the soluble fraction, and the presence of a phosphorus-rich fraction in wheat starch was postulated.

In the extensive literature on the phosphorus compounds of starches (summarized with many references by Lampitt *et al.* 1941; Lampitt, Fuller & Goldenberg, 1948), the consensus of opinion appears to be that, whereas in potato starch the phosphorus is present as an amylo-phosphoric ester, in wheat starch it is combined as phosphatide loosely bound to carbohydrate, possibly by polar adsorption. This phosphatide, however, has apparently not been isolated and characterized, its presence being presumed by several authors on not very conclusive evidence. That wheat flour contains a phosphatide was shown by Winterstein & Hiestand (1907) who demonstrated in the gluten residue obtained on washing out starch from a dough the presence of a substance, associated with reducing sugar, which contained choline and gave β-glycerophosphoric acid on acid hydrolysis. Nottbohm & Mayer (1934)

found that most of the choline present in wheat flour remains in the starch fraction washed out of a dough; the starch contained no free choline, but 90 % of the choline present was isolated in a form soluble in benzene after the starch had been ground with pumice powder and water. (It is to be noted that the phosphatide content calculated from the choline values accounted for only 35 % of the total phosphorus present in the starch.) By acid hydrolysis of wheat starch, Posternak (1935) obtained only glycerophosphoric acid and no glucose-6-phosphate. Schoch (1942) showed that most of the fatty matter and most of the phosphorus in wheat starch were soluble in 85 % methanol or 80 % dioxan, the soluble fraction having the high phosphorus content of 2·25 %. Lehrmann (1930), by extraction of the solution resulting from the acid hydrolysis of defatted wheat starch, obtained 1 % of fatty matter consisting of fatty acids, but the aqueous solution contained no nitrogen and no glycerol. Lehrmann, who made no attempt to determine how this fatty acid was combined in the starch, later (Lehrmann, 1945) confirmed Schoch's (1942) results. He agreed with the conclusion of the latter, on the meagre evidence adduced, that the major proportion of the phosphorus in wheat was present in the form of loosely bound phosphatide, a small proportion of the phosphorus being combined directly with carbohydrate.

From the extensive literature, it appears that phosphorus is combined in the wheat berry in the following forms: (*a*) inorganic phosphate (probably potassium dihydrogen phosphate), (*b*) phytin (chiefly in bran and germ), (*c*) phosphatide, (*d*) nucleic acid (especially in germ), (*e*) associated with the starch, probably as a starch phosphoric ester, (*f*) possibly also hydrolysis products of these. It is possible that any or all of these types of phosphorus may be present in wheat starch, even if only as contaminants due to incomplete separation of the starch from the other flour constituents. It is also possible that decomposition products of these phosphorus compounds may be produced during isolation of a fraction.

Preliminary work showed that starch yielded very little soluble phosphorus and nitrogen when extracted by the normal method for phosphatides using ethanol and ether. The starch was therefore completely peptized with chloral hydrate as a preliminary step, the method of Meyer, Brentano & Bernfeld (1940) being chosen as it was claimed that degradation and hydrolysis of the starch molecule are thus avoided. The peptization was followed by precipitation with acetone, both soluble and insoluble fractions being examined.

EXPERIMENTAL

The starch used. This was prepared in the usual way by washing out from a dough made from a strong wheat flour of 70 % extraction. The starch was allowed to sediment out from the wash water, washed by decantation several times with water and finally filtered on a Büchner funnel. The starch was dried in a current of hot air at a temperature below the gelling temperature, the lumps of dried starch pulverized and passed through a 48-mesh silk sieve. Microscopical examination showed that very few swollen and burst grains were present. Two samples of starch used contained approximately 8 % moisture, and (in the dry solids) 0·08 % P and 0·11 % N (N/P, 3·0/1), and 0·07 % P and 0·08 % N (N/P, 2·6/1), respectively. Starch prepared by the method used in this investigation invariably contains free phosphate. In the present case, 14 % of the total P in the starch was extractable by shaking for 1 min. with water at room temperature, and this fraction was almost entirely inorganic P. This 'adventitious P' has been borne in mind during this study; it is considered that its presence does not affect the main conclusions reached.

Methods of analysis. Nitrogen was determined by the micro-Kjeldahl procedure using Cu as catalyst; phosphorus by the Kuttner & Lichtenstein (1932) method after destruction of organic matter with H_2SO_4 and H_2O_2 (except where otherwise mentioned N and P values are calculated as percentages of the respective total N and P contents of the original starch and N:P ratios are calculated in terms of gram atoms); inorganic P by colorimetric estimation after precipitation with magnesia mixture.

Choline was determined where there was sufficient material, using the colorimetric reineckate method of Engel (1942). Two modifications in this method were found to be desirable: (1) hydrolysis with baryta for 3 hr. instead of 2 hr., (2) washing the reineckate precipitate with 3 × 2 ml. portions of ice-cold water according to the method of Ramsay & Stewart (1941); it was established that under these conditions the solubility of the precipitate was only 6 mg./100 ml. so that the loss on washing was negligible. When the acetone solution of the precipitate was unsuitable for colorimetric measurement of the colour (e.g. if the colour was too weak or the solution slightly turbid), the amount of choline present was calculated from the N content (micro-Kjeldahl) as described by Ramsay & Stewart (1941); it was found that the colorimetric and N methods gave identical results.

Glycerol was determined by the periodate procedure of Ramsay & Stewart (1941). To obtain satisfactory results with this method, it was found essential to proceed under rigidly controlled conditions and to observe the following precautions: (*a*) not more than 6 ml. of the baryta hydrolysate of the phosphatide should be taken when the volumes of reagents specified by Ramsay & Stewart are used; (*b*) the time of oxidation should be exactly 5 min.; (*c*) distillation of the formaldehyde produced should continue until the solution is almost dry before water is added prior to the second distillation; (*d*) the liquid must remain slightly alkaline during distillation to avoid evolution of formic acid (also formed on oxidation) which interferes with the subsequent colorimetric determination of the formaldehyde.

Peptization in chloral hydrate

The general procedure was as follows: 9 g. portions of starch were each dissolved by heating to 80° with 300 ml. 33 % (w/v) aqueous chloral hydrate solution (previously brought to pH 7·0 by addition of a little $NaHCO_3$) and the whole maintained at 80° for 30 min. Microscopical examina-

tion showed that the starch grains were then completely dispersed. The starch was recovered as a fine precipitate by blowing the hot solution through a fine jet into 600 ml. of dry acetone which was vigorously stirred. The solution was decanted off and the precipitate washed by decantation with 2 × 200 ml. portions of dry acetone, finally filtered on a Büchner funnel and dried at 95–100° in a steam oven. The weight of precipitate was practically equal to that of the original starch taken.

The acetone solution and washings (referred to later as 'acetone filtrate') were combined and concentrated to small bulk (50 ml.) under reduced pressure at a temperature below 60°. The concentrate, which contained chloral hydrate, was treated with 50 ml. water and again concentrated as before, whereby a small part of the chloral was removed. Further treatment to remove chloral hydrate by distillation with water was not carried out owing to the possibility of hydrolysis or other decomposition of P compounds.

In three such experiments the acetone filtrate contained on the average 60 % of the original phosphorus and 45 % of the nitrogen of the starch, and the N/P ratio was materially lower than that of the starch, indicating that fractionation of the P compounds had been effected.

Fractionation of the phosphorus compounds in the acetone filtrate

Five fractionations were carried through on the acetone filtrates obtained from 18 g. portions of the wheat starch using the procedure shown in Fig. 1. The process was carried out in a quantitative manner and samples of the various fractions were taken for determinations of N, P and choline.

The results of one such fractionation are shown in Table 1. The separation of Fractions II and III is dependent on the relative solubilities of the material in ether and in the aqueous phase, the distribution being influenced by the amount of chloral hydrate remaining after concentration of the acetone filtrate. As a consequence, in certain experiments the bulk of the P and N was found in Fraction III, and the ratio N/P was found to be approximately 1/1 in this fraction, as for Fraction II*a* in Table 1.

From these results, namely, (*a*) the solubility of P and N in ether or chloroform (Fractions II*a* or III respectively), (*b*) a N/P ratio in this fraction approximately 1/1 and (*c*) that the N was present almost exclusively as choline, it was tentatively concluded that the acetone filtrate contained a phosphatide of the lecithin type.

In most of these experiments the ether-soluble fraction (presumably phosphatide) was insoluble in light petroleum. It is well known that the solubility of phosphatides is considerably modified by the action of light and oxygen during isolation and purification. This may have occurred in the present instance in spite of the precautions taken to effect concentration below 60° in an inert atmosphere as described by Kirk, Page & Van Slyke (1934).

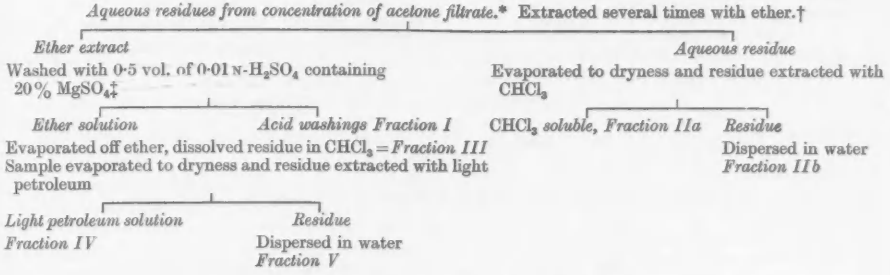
Aqueous residues from concentration of acetone filtrate.* Extracted several times with ether.†
Ether extract
Washed with 0·5 vol. of 0·01 N-H2SO4 containing 20 % MgSO4‡
Aqueous residue
Evaporated to dryness and residue extracted with CHCl3
Ether solution
Acid washings Fraction I
Evaporated off ether, dissolved residue in CHCl3 = Fraction III
Sample evaporated to dryness and residue extracted with light petroleum
CHCl3 soluble, Fraction IIa
Residue
Dispersed in water
Fraction IIb
Light petroleum solution
Fraction IV
Residue
Dispersed in water
Fraction V

Fig. 1. Fractionation of P compounds in acetone filtrate.

* All concentrations were effected *in vacuo* at temperatures below 60°, in some cases with N_2 instead of an air leak.
† As a considerable amount of emulsion formed during the first ether extraction, the mixture was allowed to stand overnight before separation. No difficulty was then experienced in subsequent extractions.
‡ This was to remove any non-phosphatide N (Kirk, 1938).

Table 1. *Fractionation of acetone-soluble phosphorus compounds*

	Original starch	Starch ppt.	Fractions from acetone filtrate					
			I	II*a*	II*b*	III	IV	V
Percentage of total P	100	37	0·6	52	2·7	1·0	Trace	Trace
Percentage of total N	100	54	1·5	24	3·8	2·1	1·2	1·7
Ratio N/P	2·6/1	3·7/1	6/1	1·15/1	2·7/1	5·6/1	—	—
Percentage of N in fraction combined as choline	—	—	—	95	—	93	—	nil

Isolation of phosphatide

The acetone filtrate prepared from a total of 112 g. of wheat starch was fractionated into the ether-soluble portion and the aqueous residue, as described in Fig. 1. These two portions were further fractionated by the method outlined in Fig. 2, when six fractions were obtained. These were dissolved in suitable solvents and samples taken for analysis. From the results shown in Table 2, it was concluded that Fraction *D* consists largely of phosphatide, as the N present was mostly in the form of choline, and the ratio of choline N/P was close to 1/1. Further, a positive test for glycerol was obtained when the method of Ramsay & Stewart (1941) was applied to this fraction.

It will be noted that small amounts of choline were present in the other fractions, but in the largest fraction (*A*) most of the N was not present as choline. The fraction which appears to be mostly phosphatide (*D*) was again soluble in $CHCl_3$ but insoluble in light petroleum.

Purification of phosphatide

A further quantity of fraction *D* was isolated by the procedure of Fig. 2 from 86 g. of wheat starch. The material was purified by solution in $CHCl_3$ and precipitated with excess of acetone, repeated three times. The product (50 mg.) was a white waxy solid. (Found: P, 3·85; N, 1·76; choline, 15·64; glycerol, 11·8. Calc. for oleopalmitophosphatide of mol. wt. 777; P, 4·0; N, 1·8; choline, 15·6; glycerol, 11·8%.)

These results indicate that a phosphatide is definitely present in wheat starch, having a composition similar to that of lecithin.

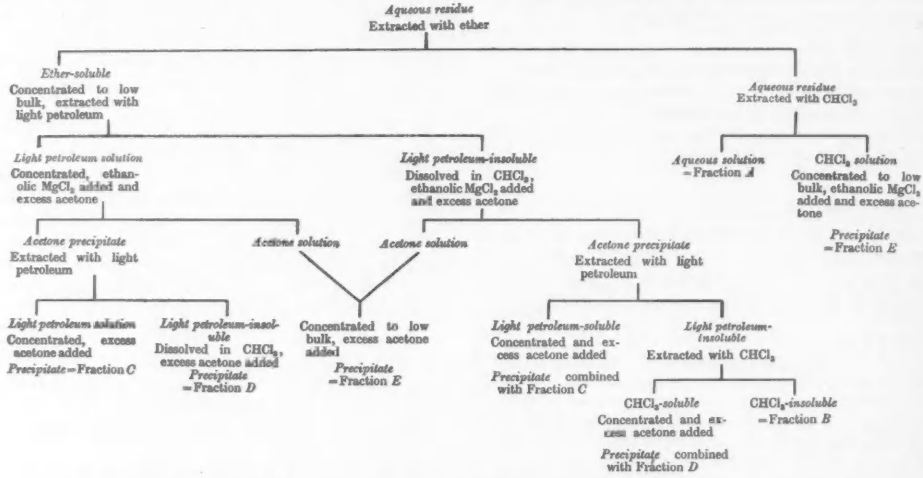

Fig. 2. Further fractionation of acetone filtrate. All acetone filtrates combined to form Fraction *F*. All acetone precipitations made at 0–5°.

Table 2. *Analysis of fractions obtained by procedure in Fig.* 2

Fraction ...	*A*	*B*	*C*	*D*	*E*	*F*
Wt. of fraction (g.)	3·96	1·83	0·011	1·118	0·79	The weight of solid matter in this fraction could not be accurately determined owing to the presence of $MgCl_2$: the fraction contained 2·3 mg. P and 4·7 mg. N of which 0·9 mg. was combined as choline; N/P, 4·5/1; choline N/P, 0·9/1
P (%)	0·158	0·433	Trace	3·13	0·132	
N (%)	0·235	0·450	Trace	1·74	0·118	
Choline N (%)	0·031	0·354	—	1·68	0·105	
Ratio N/P	3·0/1	2·4/1	—	1·2/1	2·0/1	
Ratio choline N/P	0·43/1	1·8/1	—	1·2/1	4·5/1	

Table 3. *Phosphorus and nitrogen compounds in acetone filtrate*

	Acetone precipitate	Acetone filtrate		Total in acetone filtrate (calc. as % of weight of original starch)
		Ether-soluble	Aqueous residue	
P as % of total P in starch	45	15·8	40*	P, 0·0370
N as % of total N in starch	70	13·5	21	N, 0·0240
Choline N as % of total choline N in starch	—	9·8	16·9	Choline N 0·0186
Ratio N/P	3·9/1	1·8/1	1·3/1	N/P, 1·5/1
Ratio choline N/P	—	1·3/1	1·05/1	Choline N/P 1·1/1

* In another similar fractionation, 5% of the P of the original starch was combined as inorganic phosphate in this aqueous residue.

The proportion of phosphatide in wheat starch

The P compounds in the acetone filtrate from 18 g. of wheat starch were separated merely into the ether-soluble and aqueous residue fractions in Fig. 2, and determinations of N, P and choline were made on these fractions (see Table 3). It will be noted that the ratio of total choline N to total P in the acetone filtrate is 1·1/1, from which it appears reasonable to assume that the bulk of the choline was initially present as phosphatide. From the total choline figure for the acetone filtrate, the calculated phosphatide content is 1·0 %, and from the total P content of this filtrate 0·9 %. These values, which are obviously maximum values, are comparable with the value of 0·86 % calculated from the total P contents of Fractions II*a* and III in Table 1.

Examination of the acetone precipitate (starch residue) from the chloral hydrate peptization process

The starch residue obtained by removal of the solvent from the acetone precipitate and drying in the steam oven was soluble in 0·5 N-HCl, but was precipitated on addition of an equal volume of ethanol. The ethanolic filtrate contained 10–15 % of the P of the original starch (the acid-soluble P), while approx. 30 % remained associated with the precipitate. Similar results were obtained respectively for the extract and the insoluble residue obtained on refluxing the acetone precipitate with 85 % methanol for 12 hr. Part of the acid-soluble P was combined as inorganic phosphate and part as organic phosphate (glycerophosphate or hexose phosphate), but there is a possibility that some may have been combined as nucleotide or phytin or even as phosphatide liberated by the acid from a complex with carbohydrate. A larger quantity of the acetone precipitate was therefore extracted with 0·5 N-HCl and the extract fractionated.

The acid-soluble phosphorus fraction. The dried acetone-precipitated material was moistened with a little alcohol (to avoid the formation of lumps later), 20 times the weight of 0·5 N-HCl added and the mixture shaken for 1 hr. at 15–20°. An equal volume of ethanol was added, and portions of the solution obtained on filtering off the precipitated starch were treated with (*a*) $FeCl_3$ after neutralization and (*b*) excess of $Ba(OH)_2$, as in the scheme of LePage & Umbreit (1943). The Fe precipitate was materially reduced by solution in acid and reprecipitation by neutralization, so that the presence of phytin was considered to be very doubtful. The Ba precipitate contained a little inorganic P, and an organic P compound containing glycerol but no combined sugar. In all the soluble and insoluble fractions, qualitative tests for guanine, adenine and choline before and after acid hydrolysis were negative, indicating the absence of nucleotide and phosphatide. The acid-soluble P is therefore largely glycerophosphate with some inorganic P.

DISCUSSION

The data presented afford proof that phosphatide is present in wheat starch, and therefore the statements in the literature to this effect, based on inconclusive evidence, have been substantiated. The phosphorus present (approx. 0·07 %) has been divided into three main fractions: (1) approx. 50–60 % of the total phosphorus remains in solution when a chloral hydrate dispersion of the starch is precipitated with excess acetone. This soluble phosphorus is largely present as phosphatide with a little inorganic phosphate. (2) 10 % of the total phosphorus is extractable from the acetone precipitate (starch residue) by 0·5 N-hydrochloric acid, as inorganic phosphate and glycerophosphate. (3) Approx. 30 % of the total phosphorus remains associated with the starch residue. The nature of the phosphorus compound in this fraction is being investigated.

SUMMARY

1. Peptization of wheat starch in chloral hydrate solution followed by precipitation with acetone yielded an acetone solution containing 50–60 % of the phosphorus in the original starch. From this solution a fraction was separated, soluble in ether and chloroform, in which the N/P ratio was nearly 1/1 and 95 % of the N was present as choline. Part of the 'adventitious P' present in the starch was also found in this fraction.

2. By precipitation of the chloroform-soluble material, 50 mg. of a waxy substance was isolated from 86 g. of starch, having a composition agreeing with that of lecithin. The presence of a phosphatide was therefore confirmed.

3. The phosphatide content of wheat starch was approximately 1·0 % calculated from the choline content of the acetone solution mentioned above.

4. From the carbohydrate material precipitated by acetone, approximately 10 % of the phosphorus in the original starch was extracted by 0·5 N-hydrochloric acid, 30 % remaining associated with the carbohydrate.

5. The acid-soluble phosphorus was combined as inorganic phosphate (the residual 'adventitious P') and glycerophosphate.

6. Hexosephosphate and nucleotide were not present, while there was no conclusive evidence for phytic acid.

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Studies in Vitamin A

9. THE ROLE OF THE THYROID IN CAROTENE AND VITAMIN A METABOLISM

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Apart from the action of the tocopherols in preventing the oxidation of carotenoids in the intestinal lumen, not much is known of other factors which may control the absorption and metabolism of carotene and vitamin A. Attention has, however, been focused on the thyroid gland in this connexion, especially in regard to the conversion of carotene into vitamin A. Kunde (1926) noted the appearance of vitamin A deficiency in thyroidectomized rabbits fed carotene and a little later von Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) claimed that carotene appeared in the milk of thyroidectomized goats. Abelin (1933) at the same time noted that in guinea pigs the administration of thyroxine adversely affected alike the metabolism of carotene and vitamin A. The clinical aspects of the subject have been reviewed by Drill (1943).

After Abelin's (1933) work there was a long interval before the effect of the thyroid on carotene metabolism was again investigated. Drill & Truant (1947), using the remission of xerophthalmia as criterion of vitamin A production, failed to demonstrate vitamin A formation from carotene in thyroidectomized animals. An objection to this work is that the carotene was administered parenterally and there are serious doubts whether injected carotene is utilized to any great extent (see, e.g., Sexton, Mehl & Deuel, 1946). Canadell & Valdecasas's (1947) experience appears to confirm Drill & Truant's (1947) work, but Remington, Harris & Smith (1942) state that eye symptoms in thyroidectomized rats are cured by the oral administration of carotene. Di Bella (1940*a*, *b*) also found carotene effective in such animals but with 'reduced efficiency'.

Johnson & Baumann (1947), using the storage of vitamin A in the liver as the criterion of carotene conversion, found that the same dose of carotene produced less liver vitamin A in thiourea-treated animals than in controls and that, perhaps surprisingly, control rats stored less than did rats dosed with desiccated thyroid. Administration of thyroxine and thiourea together produced normal liver storage; this indicated that the action exerted by thiourea was antithyroid and that it did not reduce liver storage of vitamin A by virtue of another (unknown) pharmacological action. Kelley & Day (1948) have confirmed the findings of Johnson & Baumann (1947).

Wiese, Mehl & Deuel (1948) emphasize the importance of allowing for the growth-inhibiting power of thiouracil when the effect of the drug on carotene metabolism is assessed by means of biological assays involving the measuring of weight increments. They proved that growth inhibition was due to thiouracil *per se*, and not to its action on carotene metabolism, by demonstrating that a normal response could be elicited in thiouracil-treated animals when desiccated thyroid, but not vitamin A, was added to the diet. Wiese *et al.* (1948) overcame this difficulty with regard to the bioassay by evaluating the dose of carotene required to produce half the maximum growth obtained in the control and experimental groups. Using this technique they found that β-carotene was equally effective in both groups.

These experiments could explain the 'reduced efficiency' of carotene as a source of vitamin A noted by Di Bella (1940*a*, *b*) in thyroidectomized animals.

The present investigation was planned on the assumption that there was an *a priori* case for implicating the thyroid in the control of the conversion of carotene into vitamin A. Further, none of the investigations just discussed indicates the possible mode of action of the hormone and the antithyroid drugs. It seemed likely that one of three possibilities could explain the adverse action of thiouracil (*a*) that the enzyme converting carotene into vitamin A in the intestinal wall is inhibited, (*b*) that the stability of carotene in the intestinal tract is reduced, and (*c*) that the absorption of carotene from the lumen is inhibited.

These three possibilities have been explored; preliminary notes of part of this work have already appeared (Goodwin, 1948; Cama & Goodwin, 1949).

EXPERIMENTAL

Animals. The rats and rabbits used were kept under normal laboratory conditions. Rabbits were maintained on a mixed diet of Lever cubes and green outer leaves of cabbage, whilst rats were maintained on cubes only; water was provided for the rats.

Special diets. When on experiment, rats were fed on either Lever cubes, ether-extracted Lever cubes, or on the carotene-(vitamin A)-free diet usually used in this laboratory (Glover, Goodwin & Morton, 1948).

Rabbit experiments. Rabbits of approx. equal weight were given a daily oral dose by stomach tube for 3 weeks (excluding Sundays) of either thiouracil or desiccated thyroid supplied by Evans Medical Supplies Ltd. Blood samples were drawn from a marginal ear vein at weekly intervals during and for some time after the administration of the thiouracil and the desiccated thyroid.

The blood samples were centrifuged and the resulting plasmas (about 5 ml. each) were examined for carotene, retinene and vitamin A according to the method in general use in this laboratory (see, e.g. Goodwin & Gregory, 1948).

Rat experiments. For each experiment rats of the same sex and about equal weights were divided into groups of three. Each group was fed either a known weight of one of the basal diets or the same weight of the same basal diet to which had been added a known amount of either thiouracil, or desiccated thyroid, or a mixture of both. Each experiment was divided into three periods: one of 7 days on the experimental diet; one of 3 days on the experimental diet during which time the rats were dosed orally with either 30 or 50 μg. of crystalline β-carotene dissolved in refined arachis oil; and one of 3 days during which the animals were again fed the experimental diet only. During these experimental periods faeces were collected completely each day and stored in the ice chest. The separate faeces were mixed well, and samples taken for analysis. The analyses were always carried out in duplicate and often in triplicate; these always agreed extremely well. The carotene concentration in the faeces was determined according to a method described by Morton & Goodwin in a recent Medical Research Council report (Hume & Krebs, 1949).

Stability experiment. Colloidal solutions (30 ml.) of β-carotene containing about 5 μg./ml. were incubated at 37° for 2·5 hr. at various values of pH, either alone or with 50 μg. of thiouracil dissolved in 5 ml. of an appropriate phosphate buffer. At the end of 2·5 hr. the pigment was extracted from the aqueous phase with freshly redistilled ether; after removing the ether, the residue was dissolved in a known volume of *cyclo*hexane for spectrophotometric measurements.

RESULTS

Rabbit experiment. This experiment was planned on the assumption that if thiouracil inhibited the intestinal 'carotenase' of animals, the plasma of which normally contains no carotene (e.g. rabbits, goats), then it might be expected that the carotene would find its way across the intestinal wall and appear in the systemic blood; this apparently occurred in Fasold & Heidemann's (1933) thyroidectomized goats which produced yellow milk. As there is good evidence to suggest that the conversion of carotene into vitamin A is a two-stage process, the first step being the production of vitamin A aldehyde (retinene) which is then quickly reduced to vitamin A (Glover *et al.* 1948), a further possibility was that the conversion of carotene into vitamin A was inhibited at the retinene stage.

Table 1. *Plasma-vitamin A levels of rabbits dosed with thiouracil and desiccated thyroid*

Treatment of animals	Vitamin A in plasma (i.u./100 ml.)				
Weeks after beginning of treatment	0	1	2	3	4
Control	139	154	148	151	140
Control	113	124	120	115	120
Thiouracil, 250 mg./day for 18 days	153	167	187	159	127
Thiouracil, 250 mg./day for 18 days	149	187*	—	141	133
Thiouracil, 500 mg./day for 18 days	193*	169	170	148	165
Thiouracil, 500 mg./day for 18 days	175	171	173	149	149
Desiccated thyroid, 200 mg./day for 18 days	104	137	123	117	108
Desiccated thyroid, 200 mg./day for 18 days	—	162	180	169	165
Desiccated thyroid, 200 mg./day for 18 days	133	148	126	123	121

* Doubtful values.

The blood of rabbits maintained on a high-carotene diet and fed thiouracil were thus examined for carotene and retinene. Because of the facilities available at the time, and of its connexion with the main problem, the effect of thiouracil on the plasma-vitamin A levels was also examined. Later the investigation was extended to include the action of

desiccated thyroid as well. The weekly values of the plasma-vitamin A levels of rabbits fed varying amounts of thiouracil and desiccated thyroid are recorded in Table 1. It will be seen that neither thiouracil nor desiccated thyroid has any effect on the plasma values.

At no time during this investigation was carotene ever detected. Since carotene was consistently absent in the plasma of the thiouracil-treated animals steps were taken to detect, if possible, traces of retinene. In *cyclo*hexane $\lambda_{max.}$ for retinene is 373 mμ. (Ball, Goodwin & Morton, 1948). No selective absorption was ever noted in this region in plasma extracts. It was not possible to test for retinene using the antimony trichloride test; in order to identify the retinene band at 664 mμ. rather than just to measure the absorption at that wave length, the Hilger-Nutting visual spectrophotometer has to be used instead of the Beckman photoelectric instrument; this needed more material than was available. In order to see if any other possible vitamin A derivatives absorbing in the region 300–360 mμ. were present the Morton & Stubbs (1946) procedure was applied; a typical result is recorded in Fig. 1. After recording the direct ultra-violet absorption spectrum of the extract (curve *A*) the correction procedure was applied to determine the absorption due to vitamin A (curve *B*); the subtraction curve *C* (curve *A* − curve *B*) records the absorption not due to vitamin A. It will be seen that between 300 and 360 mμ. the absorption is entirely general and no signs are present of a selectively absorbing compound. Incidentally, the correction procedure gives results for the plasma-vitamin A levels in good agreement with those obtained using the antimony trichloride method. For example, the values obtained for two plasmas were 128 and 165 i.u./100 ml., using the correction procedure, and 132 and 159 i.u./100 ml., using the antimony trichloride test.

Stability test. Five experiments in which thiouracil was added to colloidal carotene and incubated for 2·5 hr. at pH 7, 4 or 8, failed to reveal any action of the drug on the stability of β-carotene. Fig. 2 records the results obtained in a typical experiment; there is little quantitative or qualitative difference between the absorption spectrum of β-carotene incubated alone, or with added thiouracil. A large number of similar experiments using desiccated thyroid indicated that this also had little effect on the stability of β-carotene. These experiments will be published in detail later for they form part of an investigation into the claim that thyroglobulin converts carotene into vitamin A *in vitro* (Kaplansky & Balaba, 1946).

Absorption experiment. The results of the effect of thiouracil and desiccated thyroid are recorded in Tables 2 and 3. It is plain that even under extreme conditions of absorption (no roughage in the carotene-free diet, and a large proportion of roughage in the cubes) thiouracil inhibits and thyroid promotes absorption of carotene from the rat alimentary tract. It is only when very large amounts of carotene are provided in the food that these effects disappear. They are masked because on a large intake a high proportion of the ingested carotene is excreted in any case.

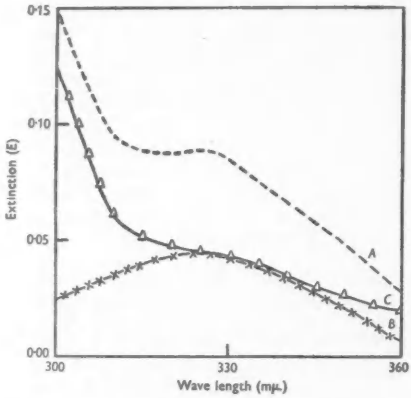

Fig. 1. The absorption spectrum of a light petroleum extract of plasma obtained from a rabbit dosed with 100 mg. thiouracil for 18 days; ----, direct absorption spectrum (curve *A*); −×−×−×−, absorption due to vitamin A (curve *B*); −△−△−△−, absorption not due to vitamin A (curve *C*).

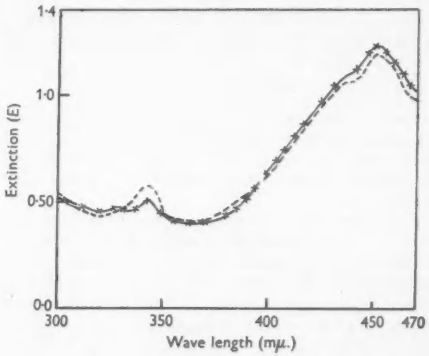

Fig. 2. The absorption spectrum of a *cyclo*hexane solution of β-carotene; ---, after the β-carotene had been incubated as a colloidal solution at pH 4 for 2·5 hr. at 37°; −×−×−×−, after the pigment had been similarly treated in the presence of thiouracil.

Table 2. *Carotene excretion by rats on various diets with and without the addition of thiouracil* (100 *mg./day*)

Diet	Duration of experiment (days)	Carotene excreted/day by a group of 3 rats (μg.) Controls	Thiouracil-fed animals	Increased excretion in the thiouracil-fed groups (%)
Ether-extracted food cubes	7	4·89	9·32	90·6
Ether-extracted food cubes	3	3·34	3·81	11·4
Ether-extracted food cubes + 30 μg. β-carotene/day	3	10·14	15·90	56·8
Carotene-free diet*	3	2·43	3·98	63·9
Carotene-free diet + 50 μg. β-carotene/day	3	7·07	9·53	34·9
Unextracted food cubes	7	60·1	57·3	[−5·0]
		58·6	54·4	[−10·7]
Unextracted food cubes + 50 μg. β-carotene	3	107	112·9	5·0
			Mean increased excretion	**30·8**

* This was during a period immediately following the administration of β-carotene which was still being excreted.

Table 3. *Carotene excretion by rats on various diets with and without the addition of desiccated thyroid* (100 *mg./day*)

Diet	Duration of experiment (days)	Carotene excreted/day by a group of 3 rats (μg.) Controls	Thyroid-fed animals	Decreased excretion of the thyroid-fed group (%)
Ether-extracted food cubes	7	4·89	3·04	37·8
Ether-extracted food cubes	3	3·34	3·41	[+2·0]
Ether-extracted food cubes + 30 μg. β-carotene/day	3	10·14	8·72	14·0
Carotene-free diet*	3	2·43	1·05	56·7
Carotene-free diet*	3	2·43	1·70†	30·0
Unextracted food cubes	7	60·1	64·9	[+8·0]
Unextracted food cubes + 50 μg. β-carotene/day	3	107·5	106·5†	1·0
			Mean decreased excretion	**18·5**

* This was during a period immediately following the administration of β-carotene which was still being excreted.
† In these experiments the dose was increased to 200 mg./day.

Table 4. *Carotene excretion by rats on a diet of ether-extracted food cubes with and without the addition of both thiouracil and desiccated thyroid* (100 *mg./day*)

Diet	Duration of experiments (days)	Carotene excretion/day by a group of 3 rats (μg.) Controls	Thiouracil + thyroid-fed animals	Decreased excretion in the group fed thiouracil + thyroid (%)
Ether-extracted cubes	7	4·89	3·19	34·7
Ether-extracted cubes	3	3·34	2·95	11·5
Ether-extracted cubes + 30 μg. β-carotene/day	3	10·14	9·83	3·1
			Mean decrease	**16·4**

It was obviously of importance to discover whether the inhibitory effect of thiouracil could be counteracted by the simultaneous feeding of desiccated thyroid. Consequently a similar experiment was carried out in which both desiccated thyroid and thiouracil were included in the basal diet. There is no doubt that the thyroid extract counteracts completely the effect of thiouracil (Table 4) and under the conditions of this experiment also exerted a positive effect in spite of the presence of thiouracil.

DISCUSSION

The results set out in this paper indicate that the thyroid does play a part in the metabolism of carotenoids. It acts, not by stimulating the conversion of carotene into vitamin A, but by controlling the intestinal absorption of carotene. The antithyroid action of thiouracil is also demonstrated by the fact that it inhibits absorption of the pigment and that this effect is counteracted by the simultaneous feeding of desiccated thyroid.

It is now possible to reconcile to some extent the conflicting reports as to the effect of antithyroid drugs on carotene metabolism. Wiese *et al.* (1948) used the bioassay to demonstrate the inability of thiouracil to inhibit the growth-promoting power of β-carotene. It is probable that at dose levels of 1 μg. of β-carotene per day the effect of thiouracil would be small enough to go unrecognized by a bioassay with such a high inherent error as that of the vitamin A assay (Gridgeman, 1944) unless a very large number of rats was used.

On the other hand, Johnson & Baumann (1947) used the liver storage of vitamin A as the criterion of efficiency of conversion of carotene into vitamin A and fed their rats at a much higher level (40–60 μg./day). Obviously, over a period of 15 days, disturbed absorption would considerably reduce the amount of carotene available for conversion and this would readily be detected in a fall in the amount of vitamin A stored in the liver; this is presumably what happened in Johnson & Baumann's (1947) experiments in which liver vitamin A was very much lower in thiourea-treated rats than in controls. The discovery of Johnson & Baumann, that rats fed desiccated thyroid stored more vitamin A than did controls, also fits in with the present observations that thyroid stimulates absorption of carotene.

It is interesting to consider briefly the effect of the thyroid on vitamin A metabolism *per se*. The results recorded in Table 1 show that, whatever the effect of the thyroid on the utilization of vitamin A (see, e.g. Baumann & Moore, 1939; Drill, 1943), neither desiccated thyroid nor thiouracil, even in large doses, has a significant effect on the plasma-vitamin A levels of rabbits. This indicates that the liver factors controlling the plasma-liver equilibrium are unaffected.

It is unnecessary to consider the mass of conflicting evidence available concerning the diagnostic use of plasma-vitamin A levels in cases of hyper- and hypo-thyroidism, for it does seem from our data that they are without value in this respect. Only in pathological conditions in which the liver-blood equilibrium is disturbed, can the determination of plasma-vitamin A levels be of any help. In other conditions the long-term uncontrolled nutritional history of a patient is the most important factor, for, because of the existence of the liver-blood equilibrium, it is only when liver stores are almost exhausted that any dramatic variations in plasma levels can be expected (Lewis, Bodansky, Falk & McGuire, 1942; Glover, Goodwin & Morton, 1947). A hypothetical example may make the argument clearer: two patients *A* and *B*, now living on diets containing only small amounts of vitamin A, develop hypothyroidism at the same time; owing to different previous nutritional histories the liver of *A* contains 25,000 i.u. and that of *B* 250,000 i.u. (a variation not beyond experience, cf. Moore, 1937). Owing to the functioning of the liver-blood equilibrium the plasma values for both *A* and *B* would fall within the normal range. Assuming a vitamin A utilization of 1250 i.u./day (Hume & Krebs, 1949) *A*'s reserves would be exhausted within 20 days and his plasma level would show a sudden drop within about 17 days; this fall could at first sight be correlated with the onset of hypothyroidism. In *B*'s case, on the other hand, 200 days would elapse before such a well-marked fall would be evident; this could not normally be correlated with the onset of hypothyroidism.

SUMMARY

1. Feeding large amounts of thiouracil to rabbits on a high carotene diet does not cause the appearance of either carotene or retinene in the blood plasma. This is taken to indicate that thiouracil does not inhibit intestinal 'carotenase'.

2. Neither thiouracil nor desiccated thyroid has any qualitative or quantitative effect on the stability of colloidal solutions of β-carotene.

3. Thiouracil inhibits and desiccated thyroid stimulates the absorption of β-carotene from the intestinal tract of rats. When administered with thiouracil, desiccated thyroid counteracts the inhibition by the drug.

4. Neither thiouracil nor desiccated thyroid has any effect on the plasma-vitamin A levels of rabbits.

5. Reasons are given for suggesting that determination of plasma-vitamin A levels are of little or no value in diagnosing thyroid dysfunction.

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Metabolism of Derivatives of Toluene

3. *o*-, *m*- AND *p*-XYLENES

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One purpose of the present study of the xylenes was to compare their excretion products in the rabbit with those found after administering the corresponding toluic acids (Bray, Thorpe & Wood, 1949) which are known to be metabolites of the hydrocarbons. There was added interest in that the xylenes are compounds with two similar potential 'centres for conjugation' (Bray, Ryman & Thorpe, 1948) and may be compared with the toluamides (Bray *et al.* 1949) which have two dissimilar potential 'centres'.

The literature contains few accounts of investigations of the fate of the xylenes in the animal body. Schultzen & Naunyn (1867) found that 'xylene' was oxidized in man and the dog to 'toluic acid' and excreted as the glycine conjugate. Curci (1892) stated that all three isomers were oxidized in the dog to the corresponding toluic acids. Filippi (1915) showed that the *o*- and *m*-isomers were similarly oxidized in the rabbit and excreted unconjugated. More recently, Kuhn & Löw (1939) isolated *p*-toluic acid as the main excretion product of *p*-xylene in the rabbit. Evidence that hydroxylation may also occur has been put forward. Curci (1892) claimed to have isolated from urine both a xylenol and a hydroxytoluic acid after the administration of each of the three isomers to the dog. Yields of 25% or more of the xylenols were reported, but were based upon weights of obviously impure fractions. The configurations of the hydroxylated metabolites were given, but were based solely upon the colour reactions of fractions or products of doubtful purity. Filippi (1915) found evidence for only a small degree of hydroxylation of *m*-xylene in the rabbit and was unable to separate any hydroxylated metabolite of *o*-xylene.

In the present investigation the effect of the three xylenes on the excretion of ether-soluble acid, reducing substances and ethereal sulphate has been studied. The chief metabolites have been isolated and characterized.

EXPERIMENTAL

Materials and methods

Diet and dosage. The rabbits and diet used were as in previous studies in this laboratory (e.g. Bray, Ryman & Thorpe, 1947). The xylenes (L. Light and Co.) were administered with water by stomach tubes. No toxic effects were observed with any of the isomers at doses up to 1·5 ml. The *m*- and *p*-isomers could be given safely in doses of 2 ml., but the *o*-isomer at this dose level occasionally caused the excretion of large amounts of reducing material. This differed from the expected metabolite in that it was not soluble in ether (cf. ether solubility of the ester glucuronide metabolite of *o*-toluic acid (Bray *et al.* 1949)). The reducing material may be a manifestation of a toxic reaction; similar observations were made with *o*-toluamide (Bray *et al.* 1949) and *o*- and *m*-acetotoluidides (Bray & Thorpe, 1948).

Methods. These were for the most part the same as those used in the study of the metabolism of the toluic acids reported previously (Bray *et al.* 1949). In some experiments the reducing power of hydrolysed urine was determined using the method described by Bray, Neale & Thorpe (1946*b*).

RESULTS

Normal excretion of metabolites

The average daily excretion of ether-soluble acid by individual rabbits ranged from 683 to 895 mg. (calculated as hippuric acid). In the majority of experiments, owing to a long delay in excretion, the percentages of the doses corresponding to the increases in ether-soluble acid excretion were calculated from single 'base-line' values, i.e. those of the days before administration of the dose. Although the results cannot be so satisfactory as those normally obtained using two or more 'base-line' values, they serve to show the general quantitative features of the oxidation of the xylenes to toluic acids.

The average daily output of reducing material by individual rabbits ranged from 147 to 195 mg. (calc. as glucuronic acid). The average percentages by

which the individual daily values differed from the weekly average base-lines used for calculation of results was ± 6 %.

The average daily excretion of ethereal sulphate for individual rabbits ranged from 25 to 39 mg. SO_3. The average percentage by which the individual daily values differed from the weekly averages was ± 9 % (± 3 mg. SO_3).

Metabolism of o-*xylene*

The results are summarized in Table 1. As with *o*-toluic acid (Bray *et al.* 1949), the ester glucuronide formed is soluble in ether and hence is included in the values for ether-soluble acid. The average total recovery according to the above results is therefore 66 %. In a few experiments at the highest dose level

Table 1. *Excretion products of* o-*xylene in the rabbit expressed as percentage of dose*

(Ranges given in parentheses.)

	Dose (g.)	No. of exps.	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether-soluble acid	0·9	3	42 (40–44)	10 (0–22)	0 (0–1)	52 (40–66)
	1·35	14	51 (29–59)	12 (0–24)	3 (0–11)	65 (47–81)
	1·8	12	37 (17–57)	13 (5–22)	3 (0–8)	54 (40–76)
	Av.		**45**	**12**	**3**	**60**
Ester glucuronide	0·9	2	22 (20–23)	0	0	22 (20–23)
	1·35	3	27 (20–35)	3 (0–5)	0	29 (20–38)
	1·8	9	30 (24–36)	2 (0–5)	0	32 (28–38)
	Av.		**28**	**2**	**0**	**30**
Ethereal sulphate	1·35	1	6	2	0	8
	1·8	4	5 (3–6)	2 (0–3)	0	6 (3–9)
	Av.		**5**	**2**	**0**	**6**

used (not included in the table) the reducing value of the unhydrolysed urine was abnormally high. It was found, however, that only a portion of the reducing material was soluble in ether, suggesting as already stated, that the remainder was probably a response to a toxic compound. The amount of ether-soluble reducing material excreted in these cases was of the same order as that normally found and recorded in Table 1.

Evidence was also obtained of the excretion of an ether-type glucuronide. The determination of ether glucuronide in the presence of ester glucuronide cannot normally be satisfactorily carried out by our method (Bray *et al.* 1946*b*). In the present instance, however, the ether solubility of the ester glucuronide permits a separation. *o*-xylene urines (as collected) were, therefore, continuously extracted with ether for 18 hr. to remove ester glucuronide and ether glucuronide was then estimated in the extracted urine. On the basis of six experiments it was estimated that about 10–15 % of the dose was excreted as an ether type glucuronide, about a third of this during the second 24 hr. after dosage. If this is added to the values given in Table 1, approximately 80 % of the dose can be accounted for.

Metabolism of m-*xylene*

The results obtained are given in Table 2. Most of the dose is excreted as ether-soluble acid which is composed of both *m*-toluic and toluric acids, the latter predominating (see p. 243) and a small amount of an ether-soluble ester glucuronide (presumably *m*-toluylglucuronide). About 4 % of the dose is hydroxylated, a finding which would appear to agree with that of Filippi (1915). No ether glucuronide was detected.

Table 2. *Excretion products of* m-*xylene in the rabbit expressed as percentage of dose* (1·74 *g. throughout*)

(Ranges given in parentheses.)

	No. of exps.	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether-soluble acid	13	59 (47–74)	14 (3–24)	8 (0–15)	81 (69–95)
Ester glucuronide	8	2 (0–4)	0	0	2 (0–4)
Ethereal sulphate	4	4 (3–4)	0	0	4 (3–4)

Table 3. *Excretion products of* p-*xylene in the rabbit expressed as percentage of dose* (1·7 *g. throughout*)

(Ranges given in parentheses.)

	No. of exps.	Day 1 (%)	Day 2 (%)	Day 3 (%)	Total excretion (%)
Ether-soluble acid	15	63 (50–76)	17 (12–24)	8 (0–16)	88 (63–109)
Ester glucuronide	10	1 (0–2)	0	0	1 (0–2)
Ethereal sulphate	4	0	0	0	0

Metabolism of p-*xylene*

Table 3 summarizes the results obtained. Virtually the whole of the dose which can be accounted for is excreted over 3 days as ether-soluble acid, the nature of which is described below. No ether glucuronide was detected.

QUALITATIVE EXPERIMENTS

o-*Xylene*. Continuous ether extraction of *o*-xylene urines gave a dark brown syrup from which *o*-toluic acid was readily isolated. A typical yield was 1·0 g. from total doses of 6 ml. (5·3 g.) *o*-xylene. Toluene fractionation of the ether-soluble material did not yield any *o*-toluric acid, but in one experiment on a larger scale (total dose 18 ml. *o*-xylene) evaporation at ordinary temperature of the mother liquors remaining after the removal of *o*-toluic acid gave 50 mg. *o*-toluric acid, m.p. 164° (Gleditsch & Moeller (1889) found m.p. 162·5°). (Found: N 6·7. Calc. for

Table 4. *Metabolism of xylenes, toluamides and toluic acids in the rabbit*

Compound	Percentage excreted as: Ether-soluble acid less ester glucuronide	Ester glucuronide	Ethereal sulphate	Total percentage accounted for	Percentage isolated* as: Glycine conjugate	Toluic acid	Other oxidized form
o-Xylene	30	30	6	80	0·3	15	—
o-Toluamide	—	—	0	20	0	0	20
o-Toluic acid	4	73	0	77	0	0	0
m-Xylene	79	2	4	85	36	2	—
m-Toluamide	85	9	0	94	25	6	28
m-Toluic acid	67	26	0	93	23	17	0
p-Xylene	87	1	0	88	42	2	—
p-Toluamide	70	4	0	74	43	3	0·4
p-Toluic acid	84	14	0	98	46	3	0

* These values were those isolated from 24 hr. urine, so that amounts actually excreted are certainly greater.

$C_{10}H_{11}O_3N$: N, 7·25 %.) As in the study of *o*-toluic acid, we were unable to obtain the ester glucuronide in crystalline form, the usual methods of isolation yielding only syrups which gave some *o*-toluic acid on hydrolysis. Preliminary attempts to isolate hydroxylation products were unsuccessful. Most of the xylenols and hydroxytoluic acids give little or no coloration with ferric chloride and the other phenol reagents (e.g. Folin & Ciocalteu, Millon, 2:6-dichloroquinonechloroimide) given by most of the fractions isolated are also given in some degree by normal rabbit urine. It is proposed to re-investigate this aspect of the metabolism of the xylenes after completing a study of the metabolism of the xylenols, which is in progress.

m-*Xylene*. The ether-soluble material obtained by continuous ether extraction of 24 hr. urine of rabbits which had each received *m*-xylene (1·5 ml.; 1·3 g.) was fractionated by refluxing with toluene as described for the toluic acids (Bray *et al.* 1949). The amount of *m*-toluric acid isolated from urine corresponding to a dose of 15 ml. (13 g.) *m*-xylene was 8·5 g. (36 % of the dose) and of *m*-toluic acid 400 mg. (2·4 % of the dose). No hydroxylation product has yet been isolated (see last paragraph under *o*-xylene above).

p-*Xylene*. The yields of *p*-toluic and toluric acids obtained from 24 hr. *p*-xylene urine as described above were 200 mg. and 10·0 g., respectively, from 15 ml. (13 g.) *p*-xylene, representing 1·2 and 42 % of the dose. While no hydroxylation product has yet been isolated there is evidence that 2:5-xylen-1-ol may be formed. This compound gives a colour with 2:6-dichloroquinonechloroimide which is characteristically different from that given by normal urine extracts (a brighter blue developing more rapidly) and this colour was obtained on testing the ether-soluble material obtained from *p*-xylene urine extracted as collected (pH 7·5).

DISCUSSION

Table 4 compares the results of the present investigation with those obtained with the toluic acids and amides (Bray *et al.* 1949).

The chief features of this comparison are the higher percentages of the doses of the toluic acids conjugated with glucuronic acid, the similarity of the glycine conjugation of the members of each group of isomers and the hydroxylation and ethereal sulphate conjugation of *o*- and *m*-xylenes.

The results provide a basis for assessing the roles of conjugation with glycine and with glucuronic acid in the metabolism of *m*- and *p*-toluic acids. It would appear that glycine conjugation of these acids takes place readily, even when their concentrations in blood are small, since there is little difference between the amounts of glycine conjugate derived from the acids administered as such or arising from oxidation of the xylenes or hydrolysis of the toluamides. It is reasonable to suppose that these latter processes involve a slowing down of the overall metabolism, so that the blood levels of the toluic acids would be expected to be lower after the administration of the xylenes or toluamides than after administration of the preformed acids themselves. The lag in excretion that occurs with the xylenes (Tables 2 and 3) also suggests a delayed metabolism, though it cannot be stated whether this is due to slow absorption, excretion or metabolism (absorption seems to have been complete in that no xylene or metabolite of xylene was detected in the faeces). This lag in excretion applies only to the glycine conjugates, since we found that the amounts of unconjugated *m*- and *p*-toluic acids excreted were very small and that there was no increase in glucuronide excretion after the first 24 hr. These facts suggest that glucuronic acid conjugation of *m*- and *p*-toluic acids may be a 'shock' metabolic process, occurring only when there is a relatively high blood concentration for which the glycine conjugation mechanism alone is temporarily inadequate, e.g. due to insufficient rate of availability of glycine. Under such conditions it would be expected that glucuronide conjugation would be greater with the toluic acids, ingestion of which would presumably cause a higher blood concentration of toluic acid than would ingestion of the xylene which must first be oxidized to toluic acid. In the case of the toluamides,

if it be assumed that hydrolysis of the amide is a preliminary to conjugation, a delayed metabolism analogous to that of the xylenes and smaller conjugation with glucuronic acid would be expected. The effect is less pronounced than with the xylenes since the hydrolysis of the amide is more rapid than oxidation of the xylene, the metabolites of toluamide being completely excreted in the first 24 hr.

Further support for the view that ester glucuronide conjugation may only occur when there is a relatively high blood concentration of an acid which is readily conjugated with glycine is provided by Snapper & Salzmann's (1947) observations that in man sodium benzoate is excreted as both hippuric acid and benzoylglucuronide whilst benzoic acid is conjugated only with glycine. These authors suggest that sodium benzoate is more rapidly absorbed than benzoic acid. A higher level of blood benzoic acid would thus be achieved by ingestion of sodium benzoate and so favour glucuronide conjugation in addition to glycine conjugation. Such an explanation could also account for our previous finding (Bray, Neale & Thorpe, 1946*a*) that, although benzamide is apparently completely hydrolysed in the rabbit, less glucuronide excretion was observed with benzamide than with benzoic acid. In experiments now in progress we have also observed that both toluene and benzyl alcohol are oxidized to benzoic acid which at small dose levels are conjugated principally with glycine and to only a small extent with glucuronic acid.

The *o*-isomers show clearly the inhibiting effect of an ortho substituent upon the glycine conjugation of a benzoic acid (cf. Quick, 1932). The effect appears to be more pronounced in the rabbit than in the dog, in which Quick found 13% of *o*-toluic acid to be conjugated with glycine and 79% with glucuronic acid. (The glycine conjugation of the *m*- and *p*-toluic acids by the dog was of the same order as we found for the rabbit.) Even with *o*-xylene, which of the three ortho compounds would be expected to provide the lowest level of toluic acid in the blood, and, therefore, result in glycine rather than glucuronic acid conjugation, glycine is very small.

There was no evidence of the oxidation of both methyl groups in any of the xylenes. With the toluamides also we obtained no indication of the conversion of both potential 'centres for conjugation' to carboxyl groups, although it was clear that either of the two centres could be modified, e.g. *m*-toluamide formed both *iso*phthalamic acid and *m*-toluric acid, but no *iso*phthalic acid, free or conjugated, was detected. It would appear from the results at present available that where a molecule possesses two potential 'centres for conjugation' only one of these is modified in each molecule, although either may be modified in different individual molecules. The evidence for hydroxylation of the xylenes shows that hydroxylation may occur even when there are two potential 'centres for conjugation' in the molecule. The absence of hydroxylation of the toluic acids and amides suggests that the hydroxylation of the xylenes takes place before oxidation of the methyl group.

SUMMARY

1. The metabolism of the three xylenes in the rabbit has been studied.

2. All three compounds are mainly oxidized to the corresponding toluic acids (60, 81 and 88%, respectively). The resulting *o*-toluic acid is excreted mainly unconjugated and as an ester glucuronide (30% of the dose), but a small amount (0·3% by isolation) is conjugated. The toluic acids from *m*- and *p*-xylenes are excreted chiefly as glycine conjugates, only small amounts being excreted free or conjugated with glucuronic acid.

3. Evidence of hydroxylation has been obtained in the case of all three isomers: 6 and 4% of doses of *o*- and *m*-xylenes, respectively, are excreted as ethereal sulphate and 10–15% of *o*-xylene is probably excreted as an ether glucuronide. *p*-Xylene may give rise to a xylenol.

4. A comparison of the results of this study with those of a previous one of the toluic acids and amides enables the roles of glycine and glucuronic acid conjugation of the toluic acids to be assessed. The conclusions drawn may also apply to other aromatic acids.

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